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Zachary A. Cheviron
University of Illinois at Urbana-Champaign, cheviron@illinois.edu

Alex D. Connaty
McMaster University

Grant B. McClelland
McMaster University, grantm@mcmaster.ca

Jay F. Storz
University of Nebraska-Lincoln, jstorz2@unl.edu

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Functional Genomics of Adaptation to Hypoxic Cold-Stress in High-Altitude Deer Mice: Transcriptomic Plasticity and Thermogenic Performance

Zachary A. Cheviron,[1] Alex D. Connaty,[2] Grant B. McClelland, [2] and Jay F. Storz[3]

1. Department of Animal Biology, University of Illinois, 515 Morrill Hall, 505 S. Goodwin Avenue, Urbana, Illinois 61801; email cheviron@illinois.edu
2. Department of Biology, McMaster University, Hamilton, Ontario L8S4K1, Canada; email grantm@mcmaster.ca
3. School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska 68588; email jstorz2@unl.edu

Abstract

In species that are distributed across steep environmental gradients, adaptive variation in physiological performance may be attributable to transcriptional plasticity in underlying regulatory networks. Here we report the results of common-garden experiments that were designed to elucidate the role of regulatory plasticity in evolutionary adaptation to hypoxic cold-stress in deer mice (*Peromyscus maniculatus*). We integrated genomic transcriptional profiles with measures of metabolic enzyme activities and whole-animal thermogenic performance under hypoxia in highland (4350 m) and lowland (430 m) mice from three experimental groups: (1) wild-caught mice that were sampled at their native elevations; (2) wild-caught/lab-reared mice that were deacclimated to low-elevation conditions in a common-garden lab environment; and (3) the F_1 progeny of deacclimated mice that were maintained under the same low-elevation common-garden conditions. In each experimental group, highland mice exhibited greater thermogenic capacities than lowland mice, and this enhanced performance was associated with upregulation of transcriptional modules that influence several hierarchical steps in the O_2 cascade, including tissue O_2 diffusion (angiogenesis) and tissue O_2 utilization (metabolic fuel use and cellular oxidative capacity). Most of these performance-related transcriptomic changes occurred over physiological and developmental timescales, suggesting that regulatory plasticity makes important contributions to fitness-related physiological performance in highland deer mice.

Keywords: Ecological genomics, high-altitude adaptation, hypoxia, phenotypic flexibility, RNA-seq, thermogenesis

Both phenotypic plasticity and genotypic specialization can contribute to differences in physiological performance between populations that are locally adapted to different environments, but their relative contributions are predicted to vary according to the spatial grain of environmental variation (Levins 1968; Scheiner 1993, 1998; de Jong and Behera 2002; Sultan and Spencer 2002; Baythavong 2011). In animal species that are distributed across steep elevational gradients, physiological challenges associated with hypoxia and cold exposure increase as a positive function of altitude, and dramatic changes in these environmental stressors can occur over relatively small spatial scales. This fine-grained environmental variation across elevational gradients should be especially conducive to the evolution of phenotypic plasticity because an increased acclimatization capacity enables organisms to track changes in local trait optima (Storz et al. 2010b). Plasticity in organismal phenotypes is often mediated by transcriptional plasticity in underlying regulatory networks (Sambandan et al. 2008; Ayroles et al. 2009; Edwards et al. 2009; Harbison et al. 2009; Zhou et al. 2009, 2012), whereas genotypic specialization may result from a canalized transcriptional pro-

gram. Assessing the degree of regulatory plasticity and regulatory canalization in species with broad elevation ranges can therefore provide fundamental insights into mechanisms of physiological adaptation to changing environmental conditions.

For small, winter-active endotherms that inhabit alpine or subalpine environments, a sustained capacity for metabolic heat production is critical for survival during prolonged periods of cold exposure. At high altitude, the energetic challenges of maintaining a constant body temperature are especially acute because environmental hypoxia imposes constraints on maximal rates of aerobic thermogenesis (Hayes and Chappell 1986; Ward et al. 1995; Chappell and Hammond 2004). Consistent with these expected effects on fitness, survivorship studies of high-altitude deer mice (*Peromyscus maniculatus*) have demonstrated that naturally occurring variation in thermogenic capacity is subject to strong directional selection in the wild, with higher capacities being associated with greater survival probabilities (Hayes and O'Connor 1999).

Deer mice are distributed from sea level to elevations of >4300 m in western North America, which makes it possible to examine evolved physiological differences between conspecific populations that are native to different elevational zones (Snyder 1981, 1982, 1985; Snyder et al. 1982; Chappell and Snyder 1984; Chappell et al. 1988; Storz 2007; Storz et al. 2007, 2009, 2010a; Cheviron et al. 2012, 2013; Tufts et al. 2013).

High-altitude deer mice in the Rocky Mountains have significantly higher thermogenic capacities under hypoxia than their lowland conspecifics (Cheviron et al. 2012, 2013), and these population differences in whole-organism performance are associated with an increased capacity to oxidize lipids as a primary fuel source during aerobic thermogenesis (Cheviron et al. 2012). These whole-organism differences in lipid catabolic capacities are in turn associated with differences in the activities of enzymes that influence flux through fatty-acid oxidation and oxidative phosphorylation pathways, and with concerted changes in the expression of genes in these same pathways (Cheviron et al. 2012). However, the extent to which these fitness-related transcriptomic differences stem from regulatory plasticity is largely unknown. Because thermogenic capacity has empirically verified effects on Darwinian fitness in high-altitude deer mice (Hayes and O'Connor 1999), and because mice that are native to different elevations exhibit pronounced differences in this fitness-related measure of physiological performance (Cheviron et al. 2012, 2013), integrative studies of performance-associated transcriptional variation can be expected to yield important insights into the role of regulatory plasticity in physiological acclimatization and adaptation to high-altitude environments.

Here we report the results of common-garden experiments that were designed to elucidate the role of regulatory plasticity in physiological acclimatization and adaptation to hypoxic cold-stress in deer mice. We combine new data on genomic transcriptional profiles and metabolic enzyme activities in skeletal muscle with previously published data on thermogenic performance under hypoxia in the same experimental animals (Cheviron et al. 2013). To assess how physiological plasticity and developmental plasticity in gene expression contribute to thermogenic performance differences, we measured highland and lowland mice from three experimental treatment groups (Table 1): (1) wild-caught mice that were acclimatized to the prevailing conditions of their native habitats; (2) wild-caught/lab-reared mice that were deacclimated to low-elevation conditions in a common-garden lab environment; and (3) the F_1 progeny of the wild-caught mice that were born and reared in the common garden. Genomic transcriptional profiles and measures of metabolic enzyme activities enabled us to identify regulatory changes in specific genes and pathways that are associated with population differences in thermogenesis under hypoxia. The combined results revealed that the enhanced thermogenic performance of high-altitude deer mice is associated with changes in the expression of genes involved in angiogenesis, muscle growth, metabolic fuel use, and mitochondrial oxidative capacity. These results suggest that coping with the twin stressors of hypoxia and cold-exposure involves regulatory changes in several intersecting physiological pathways. Consistent with previous studies of altitudinal variation in gene expression (Cheviron et al. 2008), most of these performance-related transcriptomic changes occurred over physiological and developmental timescales, suggesting that regulatory plasticity makes important contributions to fitness-related physiological performance in highland deer mice.

Table 1. Summary of acclimation treatments

Treatment Groups	Description	Highland (4350 m), *n* (males/females)	Lowland (430 m), *n* (males/females)
In situ	Sampled at native elevations within 1–2 days of capture.	10 (6/4)	10 (6/4)
6-week deacclimation	Sampled after 6 weeks of deacclimation to low-elevation (360 m a.s.l.) common-garden conditions.	10 (6/4)	10 (6/4)
F_1	Progeny of highland and lowland mice born and reared under low-elevation (360 m a.s.l.) common-garden conditions.	10 (5/5)	10 (5/5)

Methods and Materials

STUDY POPULATIONS

All of the mice included in this study were derived from a pair of high- and low-altitude localities in central North America that are separated by a linear distance of 770 km and 3920 m of vertical relief (Cheviron et al. 2012, 2013; Tufts et al. 2013). Highland mice (*P. m. rufinus*) were collected on the summit of Mt. Evans, Clear Creek Co. (Colorado; 39°35′18″N, 105°38′38″W, 4350 m a.s.l., PO_2 ~ 95.6 mm Hg), and the lowland mice (*P. m. nebracensis*) were collected in the tallgrass prairie of eastern Nebraska—9-mile prairie; Lancaster Co. (Nebraska; 40°52′12″N, 96°48′20.3″W, 430 m a.s.l., PO_2 ~ 152.0 mm Hg).

ACCLIMATION TREATMENTS

Following capture, adult highland and lowland deer mice were assigned to one of two acclimation treatments to assess the effects of postnatal environmental conditions on thermogenic performance and genomic transcriptional profiles (Table 1). Mice were either measured and sampled on-site at their native elevations within 1–2 days of capture (in situ treatment; highland mice, n = 10; lowland mice, n = 10) or they were transferred from collection localities to a common-garden lab environment at the animal research facility at the University of Nebraska–Lincoln (Lincoln, NE; elevation 360 m, PO_2 ~ 153.3 mm Hg). Mice that were transferred to the common-garden (highland mice, n = 10; lowland mice, n = 10) were housed for 6 weeks with a constant temperature (25°C) and light:dark cycle (12L:12D; in situ treatment; highland mice, n = 10; lowland mice, n = 10). After the 6-week deacclimation period, these mice were subjected to the thermogenic measurements and sampling procedures described below.

To assess the effect of pre- and postnatal developmental conditions on thermogenic performance and genomic transcriptional profiles, an additional subset of wild-caught mice was used as parental stock to establish captive-bred highland and lowland lines. From these lines, we sampled 10 full-sibling F_1 progeny that were derived from a pair of wild-caught highland parents and 10 full-sibling F_1 progeny derived from a pair of wild-caught lowland parents. Once these F_1 progeny reached adulthood (75–90 days), these mice were also subjected to the thermogenic measurements and sampling procedures outlined below. This experimental design allowed us to control for two distinct sources of phenotypic plasticity in thermogenic performance: physiological plasticity during adulthood (by comparing wild-caught/lab-reared mice from high- and low-altitude that underwent the 6-week deacclimation) and developmental plasticity (by comparing the F_1 progeny of deacclimated highland and lowland mice). Altitudinal differences in thermogenic performance that persisted in the F_1 generation likely reflect genetically based population differences in thermogenic performance. All of the mice (n = 60; 10/population/treatment) were used for measurements of thermogenic performance and metabolic enzyme activities, and a subset of 44 mice was included in the analysis of transcriptomic variation.

MEASUREMENTS OF THERMOGENIC PERFORMANCE

Measurements of whole-organism thermogenic performance in this panel of mice have been previously reported (Cheviron et al. 2012, 2013). In these studies, we measured cold-induced maximal rates of oxygen consumption (VO_{2max}) using open-flow respirometry (Figure S1). The measurements made at low elevation in Lincoln were performed under normobaric conditions (~760 mm Hg) using a hypoxic heliox atmosphere (12.6% O_2, 87.4% He), which simulates the atmospheric partial pressure of O_2 (PO_2) on the summit of Mt. Evans. For the measurements made in situ on the summit of Mt. Evans under natural hypobaric conditions (~430 mm Hg), we used a heliox atmosphere with a higher percent oxygen to correct for the reduction in atmospheric pressure at high elevation (21% O_2; 79% He). At both localities, heliox gas mixtures were equilibrated to local atmospheric pressure so that all of the experimental animals experienced an equivalent level of hypoxia during the thermogenic trials, and this level of hypoxia approximated the PO_2 at our high-altitude site. We measured thermogenic capacity (VO_{2max}) as the maximum VO_2 averaged over a continuous 5-min period, and we measured thermogenic endurance as the length of time (min) that individuals maintained ≥90% of VO_{2max} during the thermogenic trials. 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 (Rosenmann and Morrison 1974). Immediately following the respirometry measurements, all of the mice (n = 10/group) were euthanized and samples of skeletal muscle (gastrocnemis) were excised and immediately frozen on liquid nitrogen. These isolated muscle samples were used in the transcriptomic analyses and measurements of metabolic enzyme activities. All experimental protocols were approved by the University of Nebraska Institutional Animal Care and Use Committee (IACUC #522).

STATISTICAL ANALYSIS OF THERMOGENIC PERFORMANCE

Body mass varied significantly among the six experimental groups, and was significantly correlated with VO_{2max} (Cheviron et al. 2013). To control for the effects of body mass on thermogenic capacity (VO_{2max}), we used a two-way analysis of covariance (ANCOVA) design with body mass as a covariate when testing for mean differences in thermogenic capacity between samples of highland and lowland mice (Packard and Boardman 1988). By contrast, thermogenic endurance was not correlated with body mass, and as result, we used a two-way analysis of variance (ANOVA) design to test for differences in ther-

mogenic endurance. Upon detection of significant ANCOVA and ANOVA main effects, we performed post hoc Tukey tests to identify significant pairwise differences between populations and treatments. These statistical analyses were performed in R. Consistent with previous studies of thermogenic performance in deer mice (Chappell et al. 2007; Cheviron et al. 2013), sex had no effect on either thermogenic capacity or endurance. Thus, both sexes were combined in all analyses.

FUNCTIONAL GENOMICS ANALYSIS

Generation and sequencing of Illumina RNA-seq libraries

We used massively parallel sequencing of skeletal muscle transcriptomes (RNA-seq; Wang et al. 2009) to quantify genome-wide patterns of gene expression for 44 of the 60 individuals used in the thermogenic trials (In situ treatment: highland mice, $n = 6$; lowland mice, $n = 6$; 6-week deacclimation treatment: highland mice, $n = 10$; lowland mice, $n = 10$; F_1 treatment: highland mice, $n = 6$; lowland mice, $n = 6$). Transcriptomic data for the mice in the 6-week deacclimation treatment were previously reported (Cheviron et al. 2012), and in this study, we report new transcriptomic data for 24 mice from the in situ and F_1 treatments.

We isolated mRNA from gastrocnemis muscle using a micro-PolyA purist kit (Ambion, Austin, TX), and generated Illumina sequencing libraries following standard protocols (available upon request). Illumina libraries were sequenced as 76nt 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. Image analysis and base calling was performed using Illumina pipeline software. The raw sequence reads have been deposited in the NCBI sequence read archive (accession numbers SRA051883 and SRA091630).

Read mapping, normalization and statistical analysis of RNA-seq data

We performed a series of sequential filtering steps to remove low-quality reads and technical artifacts stemming from library preparation. First, we removed all reads with mean Phred quality scores less than 30. Second, we trimmed low-quality bases (Phred score < 30) from these remaining high-quality sequences. Finally, we scanned reads for adaptor sequences, and if detected, they were trimmed from the sequence read. The filtering steps resulted in a final data set of nearly 285 million sequence reads, with an average of 6.8 million reads per individual (range = 1.8–15.8 million reads), and the trimming steps resulted in an average read length of 75.4 nt.

We estimated transcript abundance by mapping sequence reads to the *Mus musculus* genome, build 36.1, using CLC Genomics workbench. In total, sequence reads mapped to 24,447 unique genes. However, we excluded genes with less than an average of 5 reads per individual because genes with low count values are typically subject to increased measurement error (Robinson and Smyth 2007). This filtering step resulted in a final data set of 12,175 detected genes. We used the function calcNormFactors in the program edgeR (Robinson et al. 2010; Robinson and Oshlack 2010) to normalize read counts among individuals, and to control for differences in the total library size (number of total reads) among individuals. Following this normalization procedure, we tested for differences in transcript abundance between populations (highland vs. lowland), and among treatment groups (in situ, deacclimation, and F_1) using a generalized linear model (GLM) approach in edgeR. We estimated model dispersion for each gene separately using the function estimateTagwiseDisp in edgeR (McCarthy et al. 2012). We tested for genes that exhibited significant expression differences between populations and/or treatments using a GLM likelihood ratio test implemented in edgeR, and we controlled for multiple tests by enforcing a genome-wide false discovery rate (FDR) of 0.05 (Benjamini and Hochberg 1995).

Identification of suites of genes that exhibit correlated transcriptional patterns can be used to define transcriptional modules of putatively coregulated genes, and functional analyses of these modules can provide insight into the mechanistic underpinnings of complex traits (Ayroles et al. 2009). Therefore, we assessed the degree of correlation in transcript abundance among the genes with significant population or treatment effects (FDR < 0.05; $n = 1435$ genes; see results) to define transcriptional modules of correlated genes that vary in their expression patterns between populations and across treatment groups. We calculated Pearson correlation coefficients for all pairwise gene expression values, and we then used modulated modularity clustering (MMC) to identify groups of highly intercorrelated genes (Ayroles et al. 2009; Stone and Ayroles 2009). Once these modules of coexpressed genes were defined, we used a suite of functional annotation tools to identify specific "biological process" gene ontology (GO) terms associated with each module. First, we used MGI GO Term Mapper (http://www.informatics.jax.org/tools.shtml) to identify GO Slim terms associated with each gene in a given module, then we used GOrilla (Eden et al. 2009) to test for functional enrichment of specific terms within modules, and we visualized the GOrilla output using the program REViGO (Supek et al. 2011). To determine which transcriptional modules were most strongly associated with thermogenic performance, we used principal components analysis (PCA) to summarize overall module expression. The first principal component axis (PC1) accounted for 82–98% of the total gene expression variance within modules. PC1 scores were then used in linear regression analyses to test for associations between module expression and the two measures of thermogenic performance (thermogenic capacity and thermogenic endurance). Principal components analysis and linear regressions were performed in JMP (SAS, Cary,

NC), and we corrected for multiple tests using Bonferroni adjusted *P*-values.

Finally, because our previous analyses had suggested that thermogenic capacity was associated with coordinated changes in gene expression across pathways that influence metabolic fuel use and cellular oxidative capacity (Cheviron et al. 2012), we performed targeted analyses of gene expression in the β-oxidation, glycolysis, TCA cycle, and oxidative phosphorylation (OXPHOS) pathways. We used Cytoscape (Cline et al. 2007; Smoot et al. 2011) to map expression values onto these selected metabolic pathways, using pathway maps that were downloaded from the KEGG database (http://www.genome.jp/kegg/pathway.html).

Enzymatic measurements of cellular aerobic capacity

The apparent V_{max} of enzymes was measured to assess the capacity of gastrocnemius muscle for glycolysis (hexokinase, HK; pyruvate kinase, PK; phosphofructokinase, PFK; lactate dehydrogenase, LDH), β-oxidation of fatty acids (β-hydroxyacyl-CoA dehydrogenase, HOAD), the TCA cycle (citrate synthase, CS, isocitrate dehydrogenase, IDH), and OXPHOS (cytochrome oxidase, COX), using assay conditions described by Cheviron et al. (2012). All samples were measured at 37°C in triplicate and background activities were subtracted for each enzyme. Measurements were done in a 96-well format on a Spectromax Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA). We tested for statistical outliers in enzyme V_{max} values using a Grubb's test after identifying several suspect values in univariate plots. A total of four measurements were identified as outliers and removed from further analysis, with no more than one value per group being discarded (HOAD n = 1, COX n = 2, PK n = 1). After removing outliers, we tested for within-treatment altitudinal differences in enzyme V_{max} using *t*-tests performed in R. Enzyme activities for the mice in the 6-week deacclimation treatment were previously reported (Cheviron et al. 2012), and in this study, we report new data for 40 mice from the in situ and F_1 treatments.

Results

WHOLE-ORGANISM THERMOGENIC PERFORMANCE

Our previous analyses of highland and lowland deer mice revealed that two different measures of thermogenic performance—thermogenic capacity and endurance—are characterized by distinct patterns of phenotypic plasticity (Cheviron et al. 2013). Thermogenic capacities differed significantly between highland and lowland mice (ANCOVA $F_{1, 59}$ = 60.17, P < 0.0001), and among the three treatment groups (ANCOVA $F_{2, 58}$ = 21.46, P < 0.0001). Post hoc tests revealed that within each experimental group (in situ, deacclimation, and F_1), high-altitude mice had significantly higher thermogenic capacities than their low-altitude

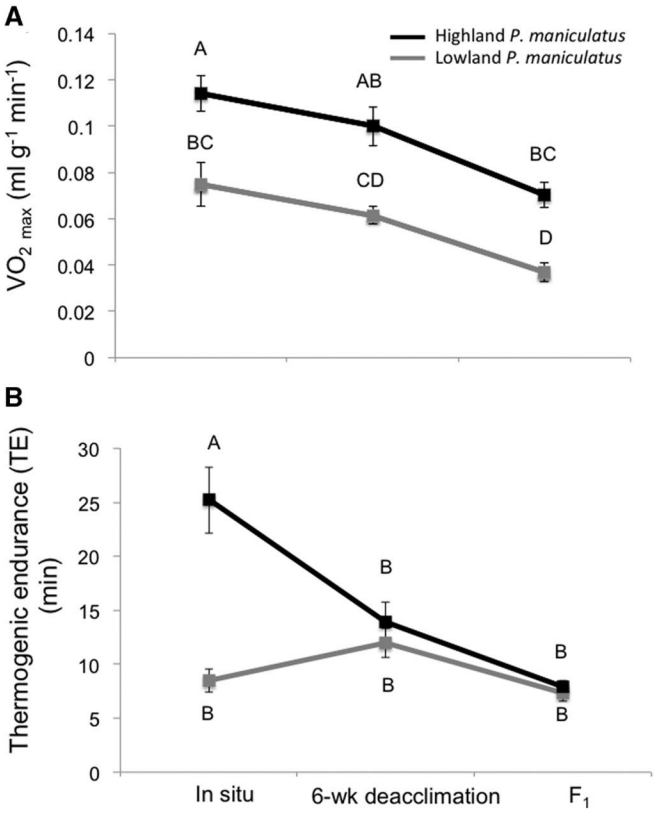

Figure 1. Altitudinal variation in hypoxic thermogenic performance in each of three experimental groups. (A) Thermogenic capacity. (B) Thermogenic endurance. Data are presented as mean ± 1 SEM. Letters denote population and treatments combinations that are significantly different from one another. Modified from Cheviron et al. (2013).

counterparts (Figure 1A), but in both groups, thermogenic capacities were reduced in the deacclimated mice and in their F_1 progeny. We found a fundamentally different pattern for thermogenic endurance. Although thermogenic endurance differed significantly between highland and lowland mice (ANOVA $F_{1, 59}$ = 22.03, P < 0.0001) and among the acclimation treatments (ANOVA $F_{1,59}$ = 15.4, P < 0.0001), this result was largely attributable to differences between the in situ groups (Cheviron et al. 2013; Figure 1B). Unlike thermogenic capacity, mean trait values for thermogenic endurance did not differ between highland and lowland deer mice in either the deacclimation or F_1 treatment groups, suggesting that the difference between highland and lowland mice in the in situ group is attributable to physiological plasticity during adulthood (Cheviron et al. 2013). Conversely, measures of thermogenic capacity for the highland mice were always significantly higher than those of the lowland mice within treatments (Figure 1A), which may reflect genetic differences in baseline thermogenic capacity that can be further modulated by physiological and developmental plasticity (Cheviron et al. 2013).

Table 2. Numbers of genes differentially expressed between populations (highland vs. lowland) and across acclimation treatments (In situ, 6-week deacclimation, and F_1 offspring)

	No. differentially expressed genes (% of total genes measured)		
Factor	FDR < 0.05	FDR < 0.1	Uncorrected $P < 0.01$
Population	153 (1.3%)	273 (2.2%)	575 (4.7%)
Treatment	1353 (11.1%)	1992 (16.4%)	1651 (13.6%)
Population + Treatment	71 (0.6%)	135 (1.1%)	216 (1.8%)

ALTITUDINAL VARIATION IN TRANSCRIPT ABUNDANCE AMONG TREATMENT GROUPS

We used a general linear model approach to identify genes that exhibited significant variation in transcript abundance as a function of altitude or experimental treatment. A total of 1435 genes (11.8% of the total number of measured transcripts) exhibited significant expression differences between populations and/or among treatments (FDR < 0.05). The majority of these genes (89.3%, 1272 genes) exhibited significant variation in transcript abundance among treatments irrespective of native altitude, and a small fraction (5.7%, 82 genes) differed between highland and lowland mice across the three treatments (Table 2). Seventy-one genes (4.9% of the variable transcripts) exhibited both population and treatment effects. These results demonstrate that 11% of detected transcripts in the skeletal muscle transcriptome were responsive to the acclimation treatments, but only a small number of genes exhibited persistent expression differences between highland and lowland deer mice in each treatment (0.7% of detected transcripts). Focusing on this subset of variable transcripts (Tables S1, S2), we calculated correlation coefficients for all pair-wise comparisons of transcript abundance to define a matrix of network connection strengths, and we then used MMC (Ayroles et al. 2009; Stone and Ayroles 2009) to identify modules of highly intercorrelated, coregulated genes. This analysis revealed a high degree of correlational structure among the variable transcripts (Figure 2).

The 1353 genes with environmentally sensitive expression (those with significant treatment effects) clustered into a total of 21 highly intercorrelated modules (Figure 2A), and 1032 of these genes (76.8%) were included in the two largest modules (T5 and T14). Because transcriptional modules are typically enriched for functionally related genes whose products interact in the same pathways, coordinated changes in the expression of these coregulated genes can suggest hypotheses about effects on pathway function (Rockman 2008; Ayroles et al. 2009). Gene enrichment analyses revealed that the two largest environmentally sensitive transcriptional modules were significantly enriched for genes involved in protein metabolism (module T5;

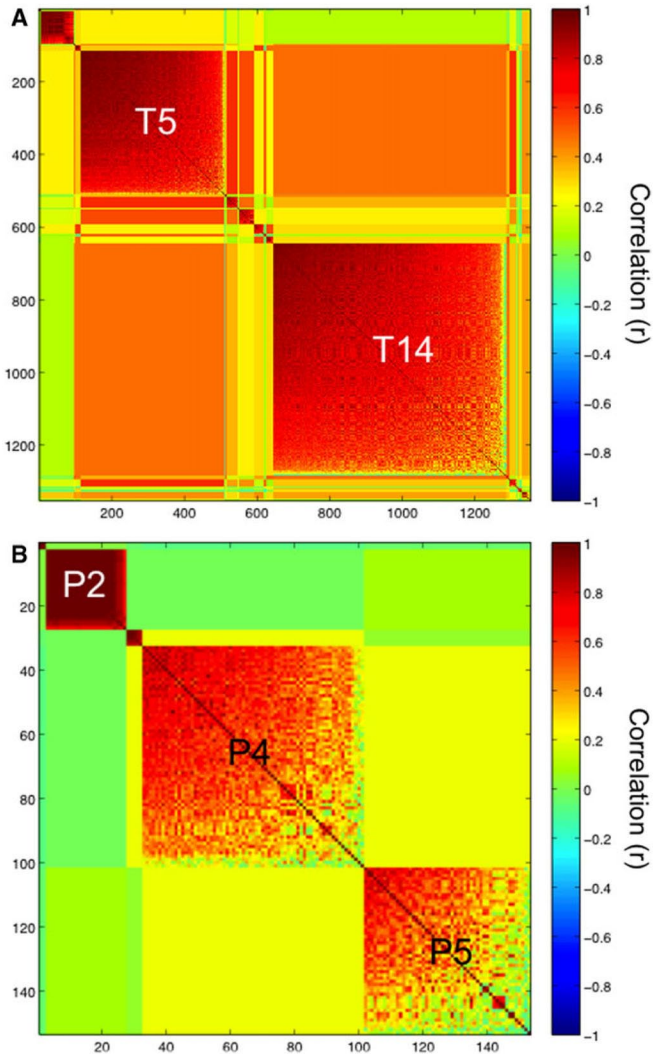

Figure 2. Correlated transcriptional modules. (A) Clustering of 1353 transcripts with significant treatment effects into 21 transcriptional modules. (B) Clustering of the 153 transcripts with significant population effects into five transcriptional modules.

$P = 3.15 \times 10^{-5}$), regulation of skeletal muscle cell differentiation (module T14; $P = 1.96 \times 10^{-4}$), vascularization (module T5; $P = 9.3 \times 10^{-4}$, module T14; $P = 1.96 \times 10^{-4}$), regulation of erythrocyte differentiation (module T14; $P = 4.10 \times 10^{-4}$), cellular responses to reactive oxygen species (module T14; $P = 5.76 \times 10^{-4}$), water transport (module T14; $P = 6.91 \times 10^{-6}$), cellular metabolic processes (module T14; $P = 5.64 \times 10^{-26}$), and gene transcription (module T14; $P = 3.38 \times 10^{-6}$). Other environmentally sensitive transcriptional modules were significantly enriched for genes involved in carbohydrate metabolism (module T3; $P = 0.001$), immune system processes (module T4; $P = 1.15 \times 10^{-5}$), muscle cell development (module T8; $P = 1.07 \times 10^{-7}$), blood vessel development (module T10; $P = 0.001$), lipid metabolism

(module T13; $P = 6.3 \times 10^{-8}$), brown fat cell differentiation (module T13; $P = 5.71 \times 10^{-4}$), leptin signaling (module T17; $P = 0.001$), lipoprotein lipid oxidation (module T17; $P = 2.0 \times 10^{-4}$), glucagon secretion (module T17; $P = 3.8 \times 10^{-4}$), and general cellular responses to hypoxia (module T18; $P = 1.2 \times 10^{-4}$).

We observed similar patterns for genes with significant population effects (Figure 2B). The 153 genes that exhibited significant expression differences between highland and lowland mice clustered into five highly intercorrelated modules, two of which (P4 and P5) contained 78.4% of the differentially expressed genes. Gene enrichment analyses revealed that modules P4 and P5 were significantly enriched for genes that participate in oxidation–reduction processes (module P5) and genes that contain Ikzf-1 transcription factor binding sites (module P4). The combined results of these analyses suggest adaptation and acclimatization to high altitude is associated with transcriptional changes that modulate several hierarchical steps in the O_2 transport cascade (Storz et al. 2010b), from tissue O_2 diffusion (angiogenesis) to tissue O_2 utilization (metabolic fuel use and cellular oxidative capacity).

ASSOCIATIONS BETWEEN TRANSCRIPTIONAL VARIATION AND WHOLE-ORGANISM PERFORMANCE

We used a combination of PCA and regression analysis to determine which transcriptional modules were most strongly associated with thermogenic performance. Because genes within transcriptional modules are, by definition, highly intercorrelated, PCA can be used to summarize expression patterns of particular transcriptional modules. The first principal component (PC1) accounted for 82–98% of the variance in gene expression across modules. We therefore used PC1 scores as an index of overall module expression to identify transcriptional modules that were significantly associated with thermogenic performance.

Of the 21 modules containing genes with significant treatment effects, four were significantly associated with either thermogenic capacity or endurance. Expression profiles for modules T16 and T21 were positively correlated with thermogenic capacity (module 16: $r^2 = 0.19$, $P = 0.002$; module 21: $r^2 = 0.093$, $P = 0.044$), and expression profiles for modules T5, T14, and T16 were positively correlated with thermogenic endurance (module 5: $r^2 = 0.14$, $P = 0.012$; module 14: $r^2 = 0.22$, $P < 0.001$; module 16: $r^2 = 0.50$, $P < 0.001$; Table 3). Only one of the modules containing genes with significant population effects was significantly correlated with thermogenic performance (Table 3). The expression of module P4 was positively correlated with both thermogenic capacity and endurance (capacity: $r^2 = 0.18$, $P = 0.003$; endurance $r^2 = 0.42$, $P < 0.001$).

Table 3. Associations between transcriptional module expression and thermogenic performance. Modules are illustrated in Figure 2, and the genes that comprise each module are presented in Tables S3 and S4. Values in bold are significant after Bonferroni correction from multiple tests. NS = uncorrected $P > 0.05$

		Thermogenic capacity		Thermogenic endurance	
Module	No. genes	r^2	P	r^2	P
P4	69	0.18	0.003	**0.43**	**<0.001**
T5	394	NS	NS	0.14	0.012
T14	640	NS	NS	**0.22**	**0.001**
T16	**20**	**0.19**	**0.002**	**0.51**	**<0.001**
T21	4	0.09	0.044	NS	NS

The modules that are associated with aspects of thermogenic performance contain genes with diverse molecular functions (Figure 3), and each module exhibited distinct patterns of change in expression among treatments (Figure 4). However, four of the five modules associated with thermogenic performance were significantly enriched for genes that influence metabolic fuel use and mitochondrial oxidative capacity. Because our previous studies of deacclimated mice suggested that the enhanced thermogenic performance of highland deer mice is associated with expression changes in genes in the fatty-acid oxidation and oxidative phosphorylation pathways (Cheviron et al. 2012), we performed a targeted analysis of transcriptomic variation across the glycolytic, lipid oxidation, TCA cycle, and oxidative phosphorylation pathways, and we also measured specific activities of key regulatory enzymes in each pathway.

INTEGRATING PATHWAY-LEVEL EXPRESSION PATTERNS WITH ENZYME ACTIVITIES

Consistent with our previous analyses of highland and lowland mice in the deacclimation treatment, pathway-level analyses revealed concerted changes in gene expression across the fatty acid β-oxidation and OXPHOS pathways across the three treatment groups, and these transcriptional patterns were largely mirrored by differences in the activities of key metabolic enzymes that serve as biomarkers for overall pathway flux (Newsholme and Crabtree 1986; Figure 5).

Of the 11 genes encoding enzymes that mediate fatty acid oxidation, five were differentially expressed ($P < 0.05$) in comparisons between highland and lowland mice in one or more acclimation treatment, and the direction of gene expression change was nonrandomly distributed across the pathway. All of the genes that were significantly differentially expressed in one or more comparisons across treatments were upregulated in the highland mice (exact binomial test, $P = 0.0625$), and within treatment groups, 80–

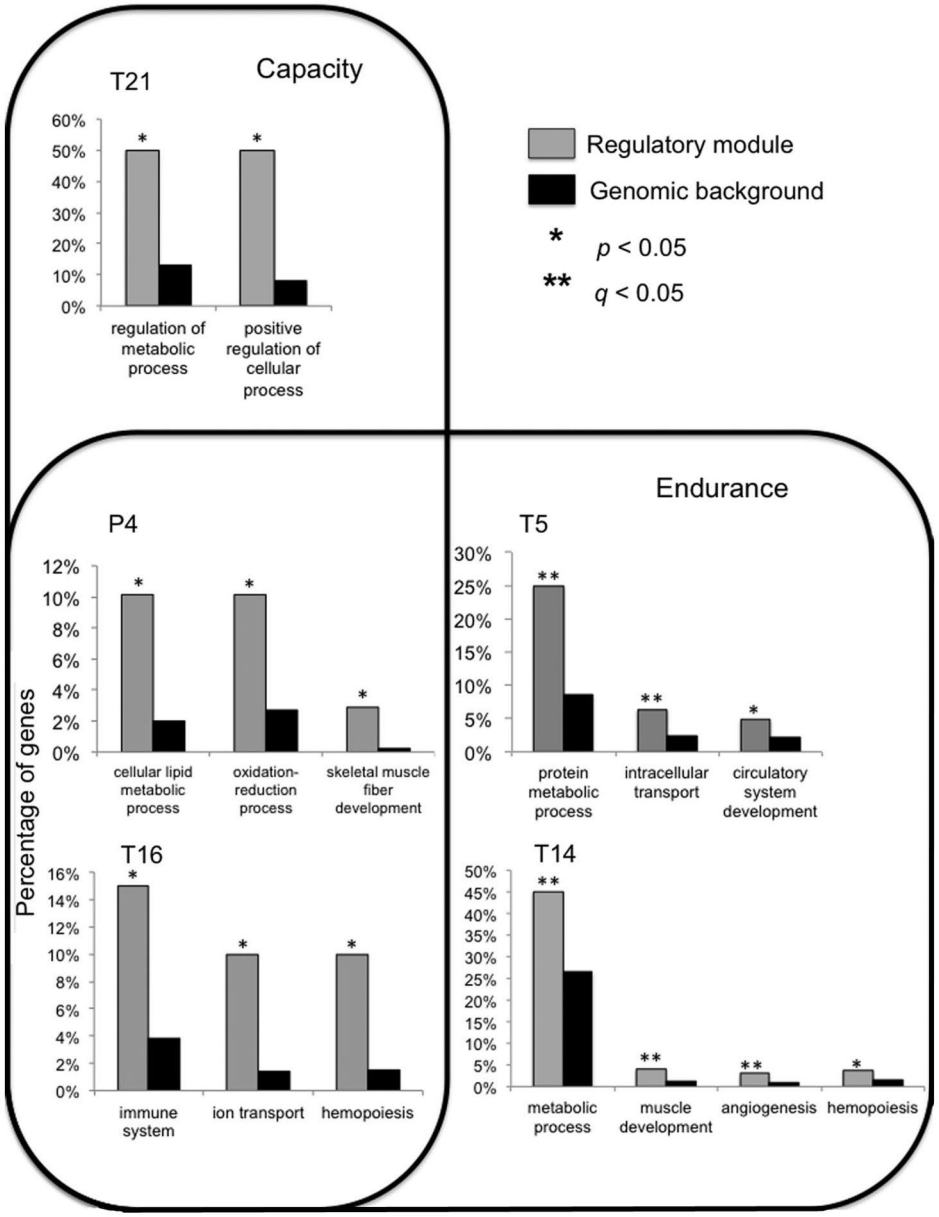

Figure 3. Transcriptional modules associated with thermogenic performance are enriched for genes in particular gene ontology categories. Categorical enrichments are shown for five separate modules that are associated with thermogenic capacity (T21), thermogenic endurance (T5 and T14), or both measures of performance (P4 and T16). For each performance-associated transcriptional module, the proportional representation of genes in different gene ontology categories is compared between the transcriptional module (gray bars) and the genome as a whole (black bars). Annotations are based on the *Mus musculus* genome. Asterisks denote gene ontology categories that are significantly enriched in the transcriptional module (*uncorrected $P < 0.05$, **FDR corrected $q < 0.05$).

100% of the genes that exhibited a >20% difference in transcript abundance between the highland and lowland deer mice were upregulated in the highland mice (Figure 5).

In the highland mice, genes involved in fatty acid oxidation were upregulated in all treatment groups and activities for β-hydroxyacyl-CoA dehydrogenase (HOAD) were also significantly elevated in the deacclimation treatment (Students *t*-test, $P = 0.007$). The altitudinal difference in HOAD activity was also marginally significant in the in situ treatment group ($P = 0.065$). These patterns suggest that highland mice in these treatment groups may have an enhanced capacity for cellular lipid oxidation (Figure 5).

We observed similar patterns of gene expression variation for the OXPHOS pathway. For the in situ and deacclimation treatments, there was a significant trend towards upregulation of OXPHOS genes in highland mice. Of 102 genes that participate in OXPHOS, 43 differed in transcript abundance by more than 20%, and of these, 72% were upregulated in highland mice in the in situ treatment (exact binomial test, $P = 0.005$). This trend toward upregulation

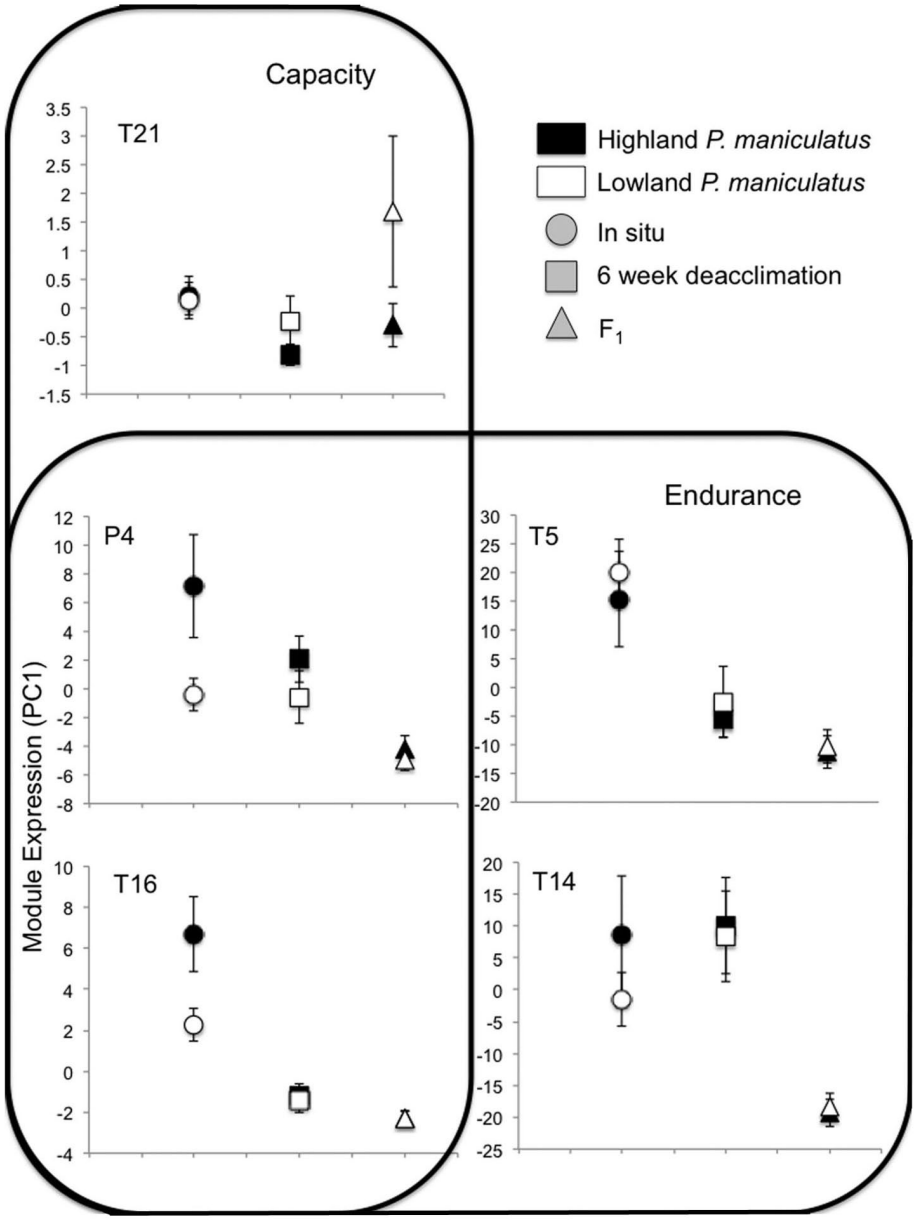

Figure 4. Altitudinal variation in the expression of performance-associated transcriptional modules in each of the 3 experimental groups. Transcriptional modules are illustrated in Figure 2, and the genes that comprise each module are presented in Tables S3 and S4.

of OXPHOS genes in highland mice was stronger in the deacclimation group, where 63 genes differed by an equivalent margin, and of these, 88.9% were upregulated in the highland mice ($P < 0.0001$). These expression patterns were mirrored by an increased activity of cytochrome c oxidase (COX) in highland mice in the deacclimation treatment group ($P = 0.039$; Figure 5). There was also a trend toward higher COX activities in the highland in situ mice, but this difference was not significant. Taken together, these results suggest that OXPHOS expression profiles in highland mice are associated with an enhanced oxidative capacity of muscle mitochondria. For the F_1 mice, however, transcript abundance for only 31 genes differed by at least 20%, the direction of gene expression change was random (exact binomial test $P = 0.28$), and there was no difference in COX activity between the progeny of highland natives and the progeny of lowland natives (Figure 5).

Also consistent with our previous studies, there was little evidence for large-scale changes in gene regulation in two other key metabolic pathways—glycolysis and the TCA cycle. With the exception of the TCA cycle in the deacclimation group, which showed a significant trend towards upregulation in the highland mice ($P = 0.03$), the direction of gene expression changes was randomly distributed across both pathways in all three treatment groups (Figure 5). Similarly, activities of glycolytic enzymes did not exhibit systematic patterns of variation between populations in the in situ or deacclimation treatments (Figure 5).

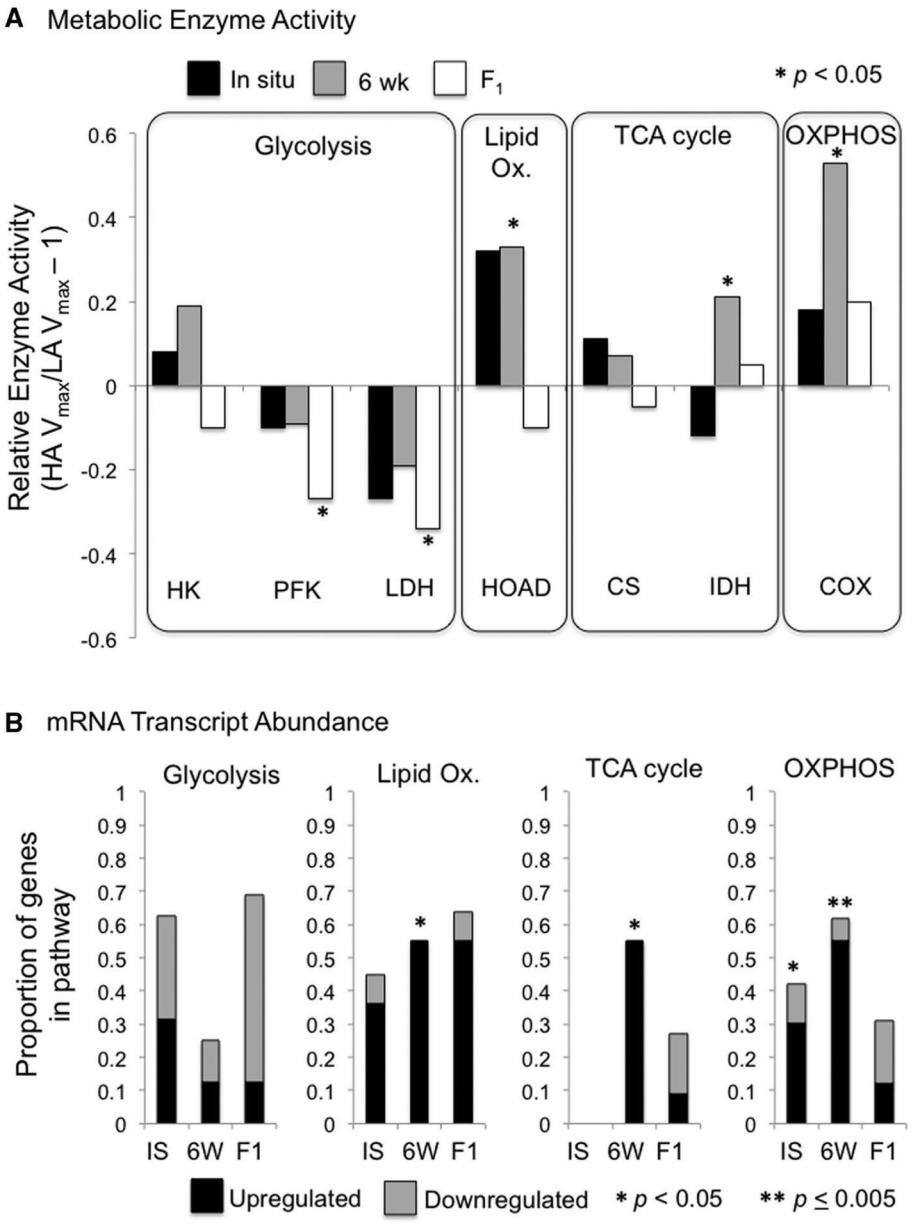

Figure 5. (A) Relative differences in skeletal muscle enzyme activities (apparent V_{max}). Positive values indicate greater mean activity in highland mice, and negative values indicate greater mean activity in lowland mice. Black, gray, and white bars represents the in situ, deacclimation, and F_1 groups, respectively. Enzymes are grouped according to metabolic pathways. Asterisks denote significant differences in enzyme activity between highland and lowland mice within an acclimation treatment (Student's *t*-test $P < 0.05$). (B) Proportion of genes in the glycolytic, fatty acid oxidation, TCA cycle, and oxidative phosphorylation (OXPHOS) pathways that are differentially expressed (>20% difference in transcript abundance) in pairwise comparisons between highland and lowland mice in each of the 3 experimental groups. The black bars and gray bars indicate genes that are up- and downregulated in highland mice, respectively. Asterisks indicate significant differences in the proportion of up- and downregulated genes (exact bionomical test; *$P < 0.05$, **$P < 0.005$).

However, there was a significant trend toward reduced glycolytic capacities in the F_1 mice that was mirrored by a nonsignificant trend toward downregulation of glycolytic genes. Finally, IDH also exhibited greater activities in highland mice in the deacclimation group ($P = 0.044$), and this increased activity was associated with significant upregulation of genes involved in the TCA cycle (Fig 5).

Ratios of the activities of metabolic enzymes provided further evidence that the enhanced thermogenic performance of highland mice is partly due to an upward scaling of oxidative metabolic potentials in skeletal muscle. CS/LDH activity ratios provide an index of the relative capacities for aerobic versus anaerobic metabolism (Hochachka et al. 1982), and elevated ratios in highland mice suggest

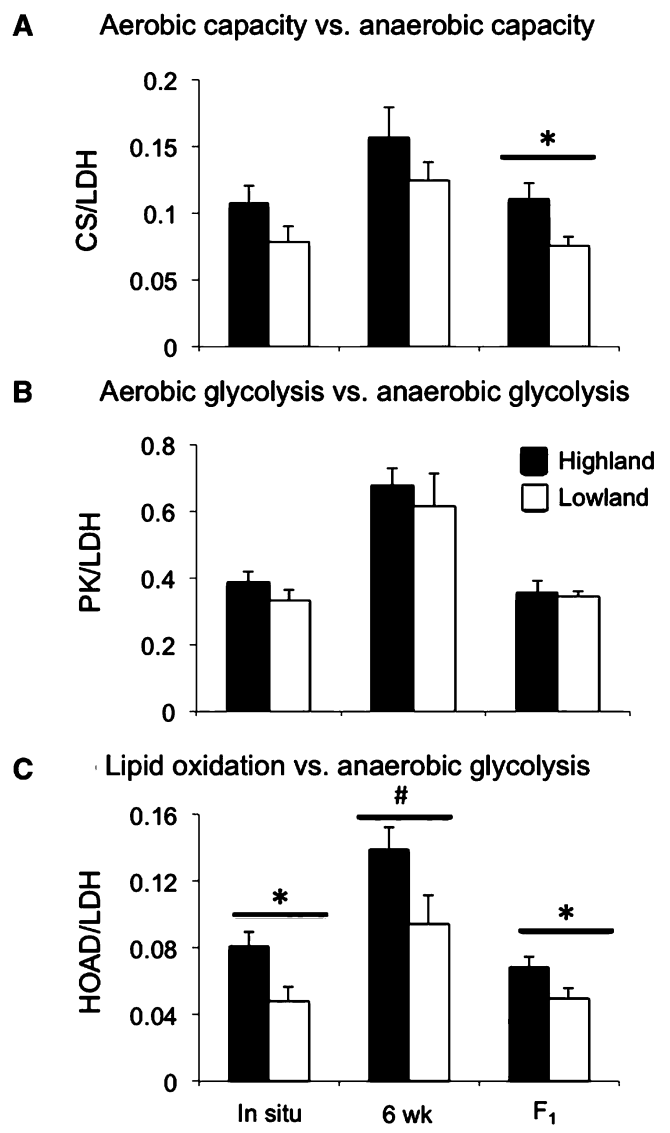

Figure 6. Ratios of metabolic enzyme activities in gastrocnemis muscle of highland and lowland deer mice in each of the 3 experimental groups. Data are presented as mean ± 1 SEM. Asterisks indicate significant differences between highland and lowland mice within treatment groups (Student's *t*-test; $^{*}P < 0.05$, $^{\#}P = 0.056$).

a greater relative aerobic capacity compared to their lowland counterparts (Figure 6). Furthermore, comparisons of PK/LDH and HOAD/LDH activity ratios suggested that the altitudinal differences in relative oxidative potentials are attributable to a greater relative capacity for lipid oxidation in highland mice rather than differences in the capacities for aerobic glycolysis (Figure 6). There was also an apparent treatment effect on these activity ratios. All three ratios were elevated in both highland and lowland mice in the deacclimation treatment compared to mice in the other two treatments (Figure 6). As indicated by the marked reduction in LDH activity in mice in the deacclimation treatment (Table S5), this result seems to be due to a reduction in anaerobic capacity in both highland and lowland mice following the deacclimation period.

Discussion

We measured metabolic enzyme activities and genomic transcriptional profiles of skeletal muscle from both highland and lowland deer mice to investigate the mechanistic underpinnings of adaptive population differences in shivering thermogenesis. The functional genomic analysis revealed significant among-treatment variation in transcript abundance in 11.7% of measured genes (Table 2), but only 0.7% of the genes exhibited constitutive expression differences between highland and lowland mice across the three experimental groups. In comparisons between highland and lowland mice, transcriptional modules that are associated with both thermogenic capacity and endurance (P4 and T16) tended to have similar expression profiles in the deacclimation and F_1 groups (Figure 4). These results suggest that much of the observed plasticity in whole-animal thermogenic performance may stem from plasticity in a small number of discrete transcriptional modules. A similar study of a broadly distributed Andean bird species (*Zonotrichia capensis*) also documented a large degree of transcriptomic plasticity in skeletal muscle in response to changes in elevation (Cheviron et al. 2008). Together, these studies suggest that regulatory plasticity may make significant contributions to the niche breadth of species that have broad altitudinal distributions.

TRANSCRIPTOMIC CORRELATES OF HYPOXIC THERMOGENIC PERFORMANCE

Although many genes exhibited significant expression differences between populations and among acclimation treatments, only a small subset of these genes was associated with variation in thermogenic performance. Genes that exhibited different expression levels between populations or among treatments clustered into a relatively small number of highly intercorrelated transcriptional modules (Figure 2). Many of these modules were enriched for genes that play a role in one or more steps of the O_2 transport cascade (Tables S3, S4), but only five modules were significantly associated with one or both aspects of thermogenic performance. These performance-associated modules were significantly enriched for genes that influence tissue vascularization, skeletal muscle growth, metabolic fuel use, and mitochondrial oxidative capacity, suggesting that elevated thermogenic performance under hypoxia is associated with simultaneous modifications of multiple, intersecting pathways (Figure 3).

Our previous analyses suggested that the elevated thermogenic capacities of highland mice were largely attributable to changes in gene expression that enhance lipid oxidation and mitochondrial oxidative capacities (Cheviron et al. 2012). The objective of this study was to assess the extent to which these performance-associated transcriptomic differences are attributable to regulatory plasticity. Highland mice in the in situ and deacclimation treatment

group exhibited elevated HOAD and COX enzyme activities, suggesting greater cellular lipid oxidation and mitochondrial oxidative capacities in skeletal muscle, and these differences were mirrored by the concomitant upregulation of genes across the lipid oxidation and OXPHOS pathways. These results suggest that variation in enzyme activities mainly stems from variation in enzyme concentration, which is largely a function of mRNA transcript abundance (i.e., transcriptional hierarchical regulation; Suarez et al. 2005; Table S6). Interestingly, traditional biomarkers of mitochondrial biogenesis (e.g., PGC-1α and Tfam) did not exhibit significant expression differences between populations or across acclimation groups after genome-wide FDR correction, suggesting that the elevated oxidative capacities of highland mice may largely stem from gene expression changes that influence the concentration of mitochondrial oxidative enzymes rather than changes in mitochondrial production. However, direct measures of mitochondrial abundance are required to confirm this. Although constitutive expression may not differ between highland and lowland deer mice, populations may exhibit differences in inducible expression in response to energetic and environmental cues.

In the case of the F_1 mice, neither HOAD nor COX activities were significantly different between the progeny of highland natives and the progeny of lowland natives that were born and reared under common-garden, low-elevation conditions. Although there was a trend toward upregulation of gene expression across the lipid oxidation pathway in the highland F_1 mice, the direction of gene expression change was random across the OXPHOS pathway. These results suggest a degree of developmental plasticity in the regulation of genes that influence cellular lipid oxidation and mitochondrial oxidative capacities, and this plasticity likely makes important contributions to thermogenic capacity at high elevation. Consistent with this idea, there was no difference in thermogenic capacity between the in situ lowland mice and the F_1 highland mice, nor was there a difference between these two groups in the activities of any of the aerobic enzymes (Table S5).

Despite the distinct patterns of phenotypic plasticity for thermogenic capacity and endurance (Figure 1), environmentally induced changes in both performance measures were associated with expression changes in the same transcriptional modules (Table 3). Although smaller modules were unique to either thermogenic capacity or thermogenic endurance, these performance-associated modules were enriched for similar metabolic gene ontology terms (Figure 3). Thus, there is little evidence that functional uncoupling of thermogenic capacity and endurance stems from differences in the transcriptional program in skeletal muscle. Instead, functional uncoupling of these performance measures may stem from plasticity in other steps of the O_2 transport cascade, differences in substrate availability, or nonshivering components of thermogenic performance.

Our combined results suggest that high-altitude adaptation and acclimatization 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 characterized by an enhanced capacity for fatty acid oxidation during exercise in normoxia (Bjorntorp 1991; McClelland et al. 1994; Henriksson and Hickner 1996; Bangsbo et al. 2006; Weber 2011; Templeman et al. 2012), and the changes in lipid oxidation and aerobic capacities across the acclimation treatments mirror physiological changes associated with cold exposure and winter acclimatization in rodents (Wickler 1981; Vaillancourt et al. 2009). Effectively allocating fuel substrates for oxidative metabolism is especially critical at high elevation. Relative to carbohydrates, the oxidation of lipids produces a higher overall yield of ATP per unit of fuel at the expense of increased O_2 consumption. The stoichiometric advantage of carbohydrate metabolism under O_2 deprivation has led to the suggestion that a shift in metabolic fuel selection in favor of carbohydrates may represent a general feature of high-altitude adaptation (Hochachka 1985). Indeed, high-altitude human populations (Sherpas and Andean Quechuas) exhibit enhanced glucose uptake, and a greater reliance on glucose for ATP production in cardiac muscle while at rest (Holden et al. 1995; Hochachka et al. 1996). Similarly, high-elevation leaf-eared mice (*Phyllotis andium* and *Phyllotis xanthopygus*) also use proportionally more carbohydrates while resting and during submaximal exercise compared to lowland congeners (*P. amicus* and *P. limatus*; Schippers et al. 2012). Our results, however, indicate the highland deer mice employ the opposite strategy to enhance thermogenic capacity under hypoxia, increasing their capacities to oxidize lipids during aerobic thermogenesis (Cheviron et al. 2012). These differences in fuel use strategies may stem from other energetic tradeoffs beyond the efficient use of O_2. Although the oxidation of glucose yields ~15% more ATP per mole of O_2 (Brand 2005), lipids make up more than 80% of the total energy reserves in mammals, and the energy density of lipids is an order magnitude greater than that of carbohydrates (Weber 2011). These energetic advantages of lipids make them the preferred fuel source during periods of sustained submaximal exercise at low elevation (McClelland et al. 1994; McClelland 2004; Weber 2011), and during high-intensity shivering thermogenesis (Weber and Haman 2005; Vaillancourt et al. 2009). Together these studies of highland mammals suggest that optimal fuel use strategies at high elevation may depend on the intensity and nature of different aerobic activities (i.e., exercise vs. thermogenesis).

As with winter-acclimatized lowland rodents, an elevated capacity for fatty acid oxidation could enhance thermogenic performance, but under hypoxic conditions at high altitude, this would require additional physiological changes to ensure adequate O_2 flux through oxidative

pathways. The elevated hemoglobin-O_2 affinity of highland deer mice safeguards arterial O_2 saturation at low PO_2, thereby preserving an adequate level of tissue O_2 delivery in spite of hypoxia (Storz et al. 2009; Storz et al. 2010a; Natarajan et al. 2013). Highland mice exhibit plasticity in hematological traits such as hemoglobin concentration that enhance blood O_2 carrying capacity (Tufts et al. 2013), and several regulatory modules were enriched for genes involved in angiogenesis that may promote an increased capacity for tissue O_2 diffusion. Changes in tissue O_2 oxygenation may help to power an enhanced capacity for lipid oxidation, underscoring the importance of integrated physiological responses to the challenges of life at high elevation.

Although we have focused on mechanisms that can enhance the capacity for shivering thermogenesis in skeletal muscle, deer mice also rely heavily on nonshivering mechanisms (Van Sant and Hammond 2008). Brown adipose tissue (BAT) is the primary site of nonshivering thermogenesis, and the size of BAT depots decreases dramatically with warm acclimation and seasonal acclimatization (Didow and Hayward 1969; Himms-Hagen 1985; Rafael et al. 1985; Klaus et al. 1988; Cannon and Nedergaad 2004). Regression of BAT depots across our acclimation treatments could lead to an increased reliance on shivering thermogenesis in the warm-acclimated mice, which would not only reduce total thermogenic capacity, but would also compound the effects of muscular atrophy associated with inactivity in captivity (Cheviron et al. 2013). Consistent with this idea, several modules associated with thermogenic performance were enriched for genes that influence muscle growth, and these modules were generally downregulated across the acclimation treatments (Figs. 3, 4). Because limb muscles like the gastrocnemis play a primary role in locomotion, the extent to which these transcriptomic changes are associated with reduced shivering thermogenesis or reduced activity in captivity is not known. Nonetheless, a similar integrative analysis of plasticity in nonshivering thermogenic performance would likely be fruitful avenue for future research.

Summary

Elucidating the mechanistic underpinnings of adaptive variation in organismal performance is a central goal of evolutionary physiology (Bennett and Huey 1990; Bennett 1991; Garland and Adolph 1991; Garland and Carter 1994; Feder et al. 2000; Dalziel et al. 2009; Storz and Wheat 2010; Whitehead 2012). Much of this work has focused on the genetic basis of canalized phenotypic traits, whereas mechanisms of physiological and developmental plasticity have received less attention in this context (but see Whitehead et al. 2011; Kvist et al. 2013). In a systems context, integrated analyses of whole-animal physiological performance and transcriptomic variation holds much promise for revealing the mechanistic underpinnings of phenotypic evolution (Dalziel et al. 2009; Storz et al. 2010b; Wheat et al. 2011; Cheviron and Brumfield 2012; Whitehead 2012). In this study, we have demonstrated that regulatory plasticity contributes to population differences in thermogenic capacity under hypoxia, a measure of whole-organism physiological performance that has a well-documented connection to Darwinian fitness (Hayes and O'Connor 1999). These results suggest that regulatory plasticity may make substantial contributions to the niche breadth of species that have broad altitudinal distributions.

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Supporting Information (files attached to repository html cover-page)

Table S1. Average log-2 counts per million reads (logCPM), likelihood ratio statistics (LR), uncorrected (*P*), and Benjamini– Hochberg corrected (FDR) *P* values for genes with significant treatment effects.

Table S2. Average log-2 counts per million reads (logCPM), likelihood ratio statistics (LR), uncorrected (*P*), and Benjamini– Hochberg corrected (FDR) *P* values for genes with significant population effects.

Table S3. Modules of correlated transcripts with significant treatment effects. Degree refers to the average correlation of a transcript with all other transcripts in the module.

Table S4. Modules of correlated transcripts with significant population effects. Degree refers to the average correlation of a transcript with all other transcripts in the module.

Table S5. Apparent *V*max for enzymes in the gastrocnemis muscle of highland (HA) and lowland (LA) deer mice in each of the 3 experimental groups.

Figure S1. Diagram of respirometry setup