

1 **Adaptive shifts in gene regulation underlie a developmental delay in thermogenesis in high-**
2 **altitude deer mice**

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9 Abstract

10 Aerobic performance is tied to fitness as it influences an animal's ability to find food,
11 escape predators, or survive extreme conditions. At high altitude, where low O₂ availability and
12 persistent cold prevail, maximum metabolic heat production (thermogenesis) is an aerobic
13 performance trait that is intimately linked to survival. Understanding how thermogenesis evolves
14 to enhance survival at high altitude will yield insight into the links between physiology,
15 performance, and fitness. Recent work in deer mice (*Peromyscus maniculatus*) has shown that
16 adult mice native to high-altitude have higher thermogenic capacities under hypoxia compared to
17 lowland conspecifics, but developing high-altitude pups delay the onset of thermogenesis. This
18 suggests that natural selection on thermogenic capacity varies across life stages. To determine
19 the mechanistic cause of this ontogenetic delay, we analyzed the transcriptomes of thermo-
20 effector organs - brown adipose tissue and skeletal muscle – in developing deer mice native to
21 low- and high-altitude. We demonstrate that the developmental delay in thermogenesis is
22 associated with adaptive shifts in the expression of genes involved in nervous system
23 development, fuel/O₂ supply, and oxidative metabolism gene pathways. Our results demonstrate
24 that selection has modified the developmental trajectory of the thermoregulatory system at high
25 altitude and has done so by acting on the regulatory systems that control the maturation of
26 thermo-effector tissues. We suggest that the cold and hypoxic conditions of high altitude may
27 force a resource allocation trade-off, whereby limited energy is allocated to developmental
28 processes such as growth, versus active thermogenesis during early development.

29 **Introduction**

30 Fitness in the wild is determined by suites of interacting traits that influence variation in
31 whole-organism performance. This is because performance determines an organism's ability to
32 conduct ecologically-relevant tasks such as avoiding predators, competing for resources, and
33 surviving extreme events (Huey and Stevenson 1979; Garland and Losos 1994; Irschick and
34 Garland 2001; Irschick et al. 2008; Campbell-Staton et al. 2017). Many of these ecologically-
35 relevant tasks that impinge on whole-organism performance are ultimately dependent upon the
36 capacity for aerobic metabolism, and thus, the ability to transport and utilize O₂. As such, the
37 development of the physiological machinery needed to delivery O₂ and metabolize fuels is
38 intimately tied to both postnatal juvenile survival and the future reproductive success as adults.
39 The timing of key developmental events, and responses to early environmental exposures, should
40 therefore influence the evolution of whole-organism performance (Lailvaux and Husak 2014).
41 Studies that seek to understand how performance evolves in the context of development are rare
42 but are needed to determine the causal connections between phenotype and fitness in the wild.

43 Extreme environments, such as high-altitude habitats > 3,000 meters above sea level
44 (a.s.l. Bouverot 1985), are windows into the mechanisms that shape adaptive variation in
45 performance, since the selection pressures are few in number and strong in magnitude (Garland
46 and Carter 1994). The agents of selection at high altitude - cold temperature and unavoidable
47 reductions in available O₂ (hypobaric hypoxia) - have led to clear examples of local adaptation in
48 animals that live there permanently (e.g., Beall 2007; Storz et al. 2010; Simonson et al. 2011). A
49 considerable amount of recent work on high-altitude adaptation has been conducted on the North
50 American deer mouse (*Peromyscus maniculatus*) owing to its broad altitudinal distribution,
51 ranging from sea level to >4,000 m a.s.l. (King 1968). Recent comparative physiological work
52 demonstrates that adult high-altitude deer mice from the Colorado Rocky Mountains have
53 consistently higher aerobic performance capabilities than conspecifics in the Great Plains: high-
54 altitude deer mice are capable of greater aerobic performance under hypoxia and in response to
55 extreme cold (referred to as thermogenic capacity) measured in the wild and in the laboratory
56 (Cheviron et al. 2012; Cheviron et al. 2013; Cheviron et al. 2014; Lui et al. 2015; Lau et al.
57 2017; Tate et al. 2017). Capture-mark-recapture data confirm that higher thermogenic capacities
58 are beneficial for survival at high-altitude (Hayes and O'Connor 1999). Available data suggests

59 that enhanced thermogenic capacity provides an important performance benefit in high-altitude
60 deer mice that is strongly tied to fitness during adulthood.

61 Recent work has uncovered the factors that shape adaptive variation in thermogenic
62 performance to illuminate links between physiology and fitness. This work, conducted in adult
63 deer mice, suggests that improved thermogenic performance is related to alterations to O₂
64 transport and utilization, including more effective breathing patterns (Ivy and Scott 2017), higher
65 blood-O₂ affinity (Snyder 1981; Snyder et al. 1982; Chappell and Snyder 1984; Storz 2007;
66 Storz 2016) and circulation (Tate et al. 2017), an improved capacity to oxidize lipids as fuel
67 (Cheviron et al. 2012; Cheviron et al. 2014), and a greater oxidative capacity in skeletal muscles
68 and their mitochondria (Lui et al. 2015; Scott et al. 2015; Mahalingam et al. 2017). Despite the
69 importance of these changes in adults, it is not clear how high-altitude animals have evolved to
70 survive the unique challenges of cold and hypoxic stress during development. Early post-natal
71 development, however, is critically linked to fitness: altricial rodent pups are small, immobile,
72 and reliant on limited energy supplied through maternal care, and pup mortality can be extremely
73 high in the wild (Hill 1983). Moreover, mouse pups are not born with the ability to generate heat
74 (Pembrey 1895). Independent thermogenic abilities develop as a result of the maturation of
75 thermo-effector organs, brown adipose tissue (BAT) and skeletal muscle, which permits non-
76 shivering and shivering thermogenesis, respectively (Lagerspetz 1966; Arjamaa and Lagerspetz
77 1979). It follows that if selection pressures on thermogenic performance are constant across
78 life stages, then thermogenesis should develop faster in high-altitude pups to support an
79 improved adult performance.

80 Robertson et al. (2019) however have recently demonstrated that non-shivering
81 thermogenesis (NST) is delayed by approximately 2-days in high-altitude deer mouse pups
82 compared to lowland conspecifics and a closely related, but strictly lowland species, *P. leucopus*.
83 This delay in the onset of NST is also further associated with a delay in the onset of shivering
84 thermogenesis until the normal date of weaning (Robertson and McClelland 2019; Fig. 1). The
85 authors suggest that the observed ontogenetic delay may be an evolutionary response to
86 limitations in energy production or allocation at high altitude (*e.g.*, as a result of low O₂), which
87 force a trade-off between active thermogenesis vs. other developmental functions such as
88 growth. Consistent with this interpretation, growth rates under common conditions are identical

89 between low- and high-altitude pups, despite the fact that high-altitude mothers produce larger
90 litters (Robertson et al. 2019).

91 We tested the hypothesis that the ontogenetic delay in thermogenesis is driven by
92 regulatory changes that delay the development of the primary thermo-effector organs. To do this,
93 we compared BAT and skeletal muscle transcriptomes in deer mice native to low and high
94 altitudes across the first 27 days of life. We associated variation in transcript abundances to
95 variation in thermogenic capabilities using a gene co-expression network approach that has been
96 used to link gene regulation to physiological function in variety of evolutionary contexts
97 (Whitehead 2012; Cheviron et al. 2014; DeBiase and Kelly 2016; Velotta et al. 2017; Campbell-
98 Staton et al. 2018). We show that the delay in thermogenesis in high-altitude mice is associated
99 with a delay in the expression of gene networks that function in nervous system control of, and
100 fuel/O₂ supply to, BAT, as well as aerobic metabolism and mitochondrial biogenesis in skeletal
101 muscle. Finally, using a combination of phenotypic divergence and population-genetic
102 approaches, we provide evidence that the ontogenetic delays in thermogenesis and their
103 associated regulatory changes are adaptive at high altitude. By combining these approaches, we
104 demonstrate, for the first time, that selection has altered the developmental trajectories of a
105 thermoregulatory system by acting on the regulatory control of thermo-effector organ phenotype.

106 **Results**

107 *The ontogeny of thermogenesis*

108 We reanalyzed cold-induced metabolic rate data (rates of O₂ consumption, VO₂) from
109 Robertson et al. (2019) and Robertson and McClelland (2019) to assess the ontogeny of
110 thermogenesis in deer mice native to lowland (320 m a.s.l) and highland (4,350 m a.s.l) habitats.
111 VO₂ of pups post-natal age p0-p10 was measured during exposure to a mild cold stressor (24°C),
112 and was calculated as the difference in cold-induced VO₂ compared to that of normothermic
113 (30°C) littermates (see Materials and Methods). VO₂ in these young pups was ~0 until p6 for
114 highlanders and lowlanders (Fig. 1A). After p6, cold-induced VO₂ increased in both populations,
115 but did so at a slower rate in highlanders (Fig. 1). Statistically significant population differences
116 in VO₂ were detected at p4 and p10 (Fig. 1). We measured thermogenic capacity in older pups
117 (age p14, p21 and p27) as VO₂-max during exposure to cold (4°C) in a heliox air mixture.
118 Highland deer mice exhibited a significantly reduced thermogenic capacity at p14 compared to

119 lowlanders (~6 ml O₂ per gram per hour, on average), whereas by p21 thermogenic capacities
120 between populations were nearly equivalent (Fig. 1B). Although there is a trend towards
121 highlanders surpassing lowlanders by p27, this result was not significant ($p=0.2$).

122 *Transcriptional correlates of thermogenesis*

123 We sequenced the transcriptomes of thermo-effector organs, brown adipose tissue (BAT)
124 and skeletal muscle, in order to assess regulatory mechanisms underlying evolutionary changes
125 in the ontogeny of thermogenesis at high-altitude. Principal components analysis (PCA) of
126 transcriptome-wide patterns of gene expression revealed separation between lowlanders and
127 highlanders, and across ontogeny, in BAT and skeletal muscle (Fig. 2). For BAT, highlanders
128 and lowlanders were distinguishable along PC1, which explained 12% of total variation in
129 expression. By contrast, PC2, which explained 10% of the variance, distinguished post-natal age
130 (Fig. 2A). We observed similar separations in muscle samples, although in this case populations
131 were distinguishable along PC1 (20% variance), and post-natal age distinguishable along PC2
132 (10% of variance). 95% confidence ellipses were drawn around each population demonstrating
133 that populations were entirely (skeletal muscle, Fig 2B) or almost entirely (BAT Fig. 2A) non-
134 overlapping in PC1 and PC2 space.

135 Weighted gene co-expression network analysis (WGCNA) of BAT samples across p0-
136 p10 yielded 38 modules ranging in size from 61 to 1,073 genes (See Supplemental Table S2 for a
137 full list of module assignments). A total of 1,588 out of 11,192 genes could not be assigned to
138 any module (Module B0). We found ten modules exhibiting statistically significant associations
139 between module eigengene and cold-induced VO₂, after correction for multiple testing (Table 1).
140 Of the ten VO₂-associated modules, six exhibited significant population effects on module
141 eigengene (Table 1). Two of these (modules B11 and B35) were not significantly enriched for
142 GO terms or were enriched for terms unrelated to thermogenesis (e.g., “drug metabolism”; see
143 Table S4 for the full list of enriched terms). Modules B4 and B12 (Table 1) were both
144 significantly enriched for genes that encode ribosomal proteins (Supplemental Table S4), which
145 may indicate differences in protein translation between populations. No significant interaction
146 effects were detected for any VO₂-associated module (Table 1).

147 Modules B3 and B36, by contrast, were positively associated with cold-induced VO₂
148 (Fig. 3A-B), expressed at significantly lower levels in highlanders across development (Fig. 3B-

149 C), and exhibited significant enrichment for functions that reflect a delay in the development of
150 BAT in highlanders (Fig. 3E-F). Module B3 was enriched for functional terms related to fatty
151 acid metabolism in the mitochondria, including the GO Biological Process term “fatty acid
152 metabolic process”, the GO Cellular Component term “mitochondrion,” the KEGG pathway
153 “fatty acid degradation (Fig. 3E), and Reactome and WikiPathways terms “fatty acid
154 metabolism” and “fatty acid beta oxidation (Supplementary Table S4).” Module B36 was
155 enriched for terms related to the vascularization of BAT and the development of its neural
156 circuitry (Fig. 3F): vascularization terms include the GO Biological Processes “blood vessel
157 development” and “vasculature development,” as well as the KEGG pathway “VEGF signaling,”
158 which is the major pathway regulating new blood vessel growth. Indeed, *Vegfa* exhibits
159 significantly lowered expression in highland compared to lowland mice (Fig. 3F inset), though it
160 was not assigned to module B36 (Supplemental Table S2). Finally, we detected enrichment of
161 the KEGG pathway “axon guidance,” which represents a key stage in the development of
162 neuronal networks. Fig. 3B-C reveal that expression values for both of these modules increased
163 from birth to post-natal day 10, and that expression was consistently higher among lowlanders at
164 nearly every time point ($p < 0.001$ population effect for both modules).

165 We performed WGCNA on skeletal muscle transcriptomes from lowland and highland
166 deer mice sampled at postnatal days 14, 21, and 27. WGCNA identified seven modules ranging
167 in size from 92 to 2,878 genes (Table 2). A total of 3,529 out of 11,104 genes could not be
168 assigned to any module (M0; Supplemental Table S2). Although individual-level thermogenic
169 capacity data do not exist for the samples sequenced, we did detect significant correlations
170 between module eigengene and thermogenic capacity at the level of population and age for two
171 modules, M2 ($r = -0.94$; $p = 0.006$) and M6 ($r = 0.98$; $p < 0.001$; Fig. 4A; Table 2). Although we
172 detected an overall effect of postnatal age, but not population, on both of these modules (Table
173 2), expression followed a pattern that closely resembled population-level variation in
174 thermogenic capacity. For module M6 in particular, as with thermogenic capacity (Fig 1B),
175 expression was lower in highland compared to lowland deer mice at p14 ($F_{1,4} = 27.7$; $p = 0.006$),
176 but no different from lowlanders at p21 or p27 ($p > 0.05$; Fig. 4B). The opposite was true of M2,
177 whereby module expression at p14 was significantly higher in high-altitude mice ($F_{1,4} = 27.7$;
178 $p = 0.02$).

179 Functional enrichment analysis of VO₂-associated skeletal muscle modules revealed
180 enrichment of a wide variety of biological functions (Supplemental Table S4). Module M2 for
181 example was enriched for pathways that function in the development of muscle blood supply,
182 including the GO Biological Process terms “angiogenesis, blood vessel morphogenesis, and
183 vasculogenesis.” This result may reflect an initiation of blood vessel development that eventually
184 leads to a greater production of capillaries in highlanders by p21 (Robertson and McClelland
185 2019). Indeed, along with a greater proportion of oxidative fiber types, increased capillary
186 density is thought to be an adaptive advantage at high-altitude that contributes to improved
187 aerobic performance capabilities in adult mice (Lui et al. 2015; Scott et al. 2015; Nikel et al.
188 2018).

189 By contrast to M2, module M6 was enriched for functions related to energy metabolism
190 and the generation of ATP, and thermogenesis, including the KEGG terms “oxidative
191 phosphorylation, thermogenesis, citrate cycle, and glycolysis/gluconeogenesis (Fig. 4C).”
192 Enrichment of the GO Cellular Component terms “NADH dehydrogenase complex and
193 mitochondrial respiratory chain complex I” indicates that energy metabolism functions are
194 related to changes in the production of NADH:ubiquinone oxidoreductase/mitochondrial
195 complex I, the first protein complex in the electron transport chain. We also detected significant
196 enrichment of transcription factor binding sites for estrogen-related receptor alpha, ERR1 (Table
197 S4; *Esrra* in the *Peromyscus* genome), which is a major regulator of mitochondrial biogenesis,
198 gluconeogenesis, oxidative phosphorylation, and gluconeogenesis. Indeed, the *Esrra* gene itself
199 was assigned to module M6 and its expression at p14 is strongly downregulated in highlanders
200 relative to lowlanders (Fig. 4D). Detection of *Ppara* (peroxisome proliferator activated receptor
201 alpha) in module M6 (Fig. 4E) suggests that fatty acid oxidation may also be depressed in
202 highlanders, since this gene is a major regulator of fat metabolism in muscle (Issemann and
203 Green 1990).

204 *Evidence of selection on candidate transcriptional modules*

205 Among the transcriptional modules identified by WGCNA, three (B3, B36, M6) were
206 significantly and positively associated with variation in VO₂. These modules thus represent
207 candidate pathways underlying the delay in thermogenesis in high-altitude mice. We took a two-
208 pronged approach to determining whether developmental delays in candidate module expression

209 are adaptive at high-altitude. First, we calculated P_{ST} (an estimate of phenotypic differentiation
210 following Brommer 2011) on module eigengene values of candidate modules to test whether
211 divergence between lowland and highland populations exceeded neutral expectations as set by
212 F_{ST} (an estimate of neutral genetic differentiation). For BAT modules B3 and B36, P_{ST} exceeded
213 F_{ST} at nearly all values of c/h^2 (Fig. 5A-B); c/h^2 represents the unknown degree to which
214 phenotypes are due to additive genetic effects. Brommer (2011) assumes a null $c/h^2 = 1$, meaning
215 that the proportion of phenotypic variance that is due to additive genetic effects is the same
216 within- and between-populations. We note that this is a conservative estimation of c/h^2 as many
217 studies to-date assume $c=1$ and $h^2=0.5$ (e.g., Ghalambor et al. 2015). In our data, the 95% lower
218 confidence limit of P_{ST} exceeds average F_{ST} at $c/h^2 > 1$ for both modules B3 (Fig. 5A) and B36
219 (Fig. 5B). This result is robust to extremely conservative estimates of $c/h^2 < 1$ (Fig. 5A-B).
220 Moreover, for most values of c/h^2 , P_{ST} exceeds even the upper 95% quantile of F_{ST} (Fig. 5A-B).
221 By contrast, 95% confidence intervals for P_{ST} calculated on the B0 module (which is not
222 associated with thermogenesis) overlap with F_{ST} at all values of c/h^2 (Fig. 5C), indicating that
223 differentiation in expression of this module does not exceed neutral expectations, as expected.
224 P_{ST} calculated on VO_2 across p0-p10 did not exceed F_{ST} , except when we restricted it to p10
225 (Supplementary Fig. 2), the time point under which VO_2 is significantly lower in highlander
226 compared to lowlanders (Fig. 1A).

227 We found that P_{ST} on the skeletal muscle module M6 did not exceed neutral expectations:
228 although observed P_{ST} does exceed F_{ST} , lower 95% confidence limits overlap entirely with zero
229 (Supplemental Fig. S1). We note that P_{ST} does not tend to overlap with the upper 95% limit of
230 F_{ST} when we restrict it to p14 (the time point of significant differentiation in module expression
231 and thermogenic capacity; Figs. 1B and 4B; see Supplemental Fig. S2). This pattern of
232 phenotypic differentiation is mirrored by P_{ST} on thermogenic capacity at p14, which greatly
233 exceeds the upper 95% limit of F_{ST} at all values of c/h^2 (Supplemental Fig. S2).

234 Finally, we tested whether these candidate VO_2 -associated transcriptional modules were
235 enriched for genes exhibiting signatures of positive natural selection, as measured by PBS.
236 Roughly 25% of the genes in modules B3, B36 and M6 contained SNPs with PBS values that
237 exceeded neutral expectations (Table 3). However, only B36 exhibited a significant enrichment
238 of outlier SNPs relative to the background rate (Fisher's Exact Test, $p=0.02$; Table 3). This

239 analysis demonstrates that, although all phenotype-associated modules exhibit some proportion
240 of genes under selection, only B36 contains more than was expected by chance.

241 **Discussion**

242 We explored the mechanisms that underlie a developmental delay in the onset of
243 thermogenesis in juvenile high-altitude deer mice (Fig 1). We show that the developmental
244 trajectory of the thermoregulatory system has evolved at high altitude *via* selection on the
245 regulatory systems that control the development of thermo-effector tissues, BAT and skeletal
246 muscle. Combined with the results of Robertson et al. (2019) and Robertson and McClelland
247 (2019), our work suggests that the cold hypoxic conditions of high-altitude force an alternative
248 resource allocation strategy, whereby limited energy is put into developmental processes such as
249 growth, over the development of the thermoregulatory machinery needed in heat production.

250 *A developmental delay in non-shivering thermogenesis is associated with a delay in expression*
251 *of fuel supply, oxygenation, and axon guidance pathways in BAT*

252 During early postnatal development mice rely exclusively on BAT for thermogenesis,
253 and low-altitude pups activate BAT to maintain body temperature in the face of cold by p10. Our
254 analysis suggests that an ontogenetic delay in the development of non-shivering thermogenesis in
255 high-altitude deer mice (Fig. 1A) is attributable to a delay in the nervous system innervation and
256 O₂/fuel supply lines to a maturing BAT. WGCNA of BAT revealed two transcriptional modules
257 (B3, B36) that are expressed at higher levels in lowlanders across age, but which show overall
258 higher expression in both populations (Fig 3). Both modules were significantly correlated with
259 cold-induced VO₂, suggesting that changes in the expression of genes in these modules influence
260 the development of thermogenesis. Module B3 was significantly enriched for pathways involved
261 in metabolism, particularly fatty acid degradation (Fig. 3; Table S4). This is interesting since fat
262 is the primary fuel source that powers uncoupling of cellular respiration from ATP production in
263 non-shivering heat production (Cannon and Nedergaard 2004). Indeed, the β₃ signaling cascade,
264 initiated by cold, stimulates the lipolysis of fats used in β-oxidation and the eventual uncoupling
265 of ATP synthesis from electron transport *via* activation of Uncoupling Protein 1 (*Ucp1*).
266 Although not assigned to B3, we found that expression of *Ucp1* itself was lower in highlanders
267 compared to lowlanders (ANOVA, main effect of population; $F_{1,30}=8.2$; $p=0.007$).

268 Module B36, by contrast, was enriched for two pathways that are important in O₂/fuel

269 supply and cold-activation of BAT. For example, enrichment of the vasculature endothelial
270 growth factor (VEGF) signaling pathway, and downregulation of *Vegfa* in highlanders (Fig 3B),
271 suggests a reduction in the vascularization of BAT early in the postnatal period in high-altitude
272 natives. This is notable since the VEGF cascade stimulates angiogenesis, increasing O₂ and
273 metabolic fuel delivery. Moreover, *Vegfa* itself contains two SNPs above the 99.9% PBS
274 threshold (Schweizer, Velotta, et al. 2019), suggesting that changes in the VEGF pathway are the
275 target of selection at high-altitude. We also detected enrichment of the KEGG pathway ‘axon
276 guidance,’ which represents a key stage in development in which neurons send out axons to
277 reach their correct targets. Indeed, BAT is activated by the sympathetic nervous system in
278 response to cold, which leads to the release of norepinephrine and the signaling cascade that
279 produces heat (Cannon and Nedergaard 2004).

280 A reduction in the expression of genes that participate in axon guidance suggests a
281 developmental delay in the sympathetic innervation of BAT, which would inhibit recruitment
282 and activation of this tissue during cold exposure (Robertson et al. 2019). Robertson et al. (2019)
283 found lower levels of the enzyme tyrosine hydroxylase (TH) in the BAT of high-altitude pups at
284 p10, lending support to the hypothesis that neurotransmitter synthesis and neural activation of
285 BAT tissue is delayed in highlanders; TH is the rate-limiting enzyme in norepinephrine
286 production and is present in sympathetic neurons innervating BAT. Related to the reduced
287 sympathetic activation of BAT, we found that expression of the gene *calstn3* (*Clstn3*) is
288 also downregulated in highlanders (ANOVA, main effect of population; $F_{1,30}=15.1$; $p<0.001$).
289 *Clstn3* is a known promoter of synapse formation; a recent study in house mice found that a
290 mammalian-specific form (*Clstn3β*) enhances functional sympathetic innervation of BAT (Zeng
291 et al. 2019).

292 Despite downregulation of genes involved in O₂/fuel supply and innervation of BAT,
293 Robertson et al. [2019] found that BAT mass does not differ between highlanders and
294 lowlanders, and that citrate synthase (a biomarker of mitochondrial abundance) and UCP protein
295 expression are likewise equivalent. Our results therefore suggest that, although BAT growth and
296 metabolic potential may be equal in highlanders and lowlanders, a delay in the development of
297 the innervation and O₂/fuel supply to BAT may lead an inability of highlanders to respond to
298 cold temperatures and thus mount an appropriate thermoregulatory response.

299 *A developmental delay in thermogenic capacity is associated with downregulation of energy*
300 *metabolism pathways in muscle*

301 Transcriptome analysis of gastrocnemius muscle revealed a large transcriptional module
302 (M6) that closely tracked variation in thermogenic capacity (Fig. 4). These results suggest that
303 the genes expressed in module M6 may underlie the developmental delay in thermogenesis
304 beyond p10 (Fig. 1B). The M6 module (Table 2) was enriched for a host of functions related to
305 skeletal muscle metabolism that reflect changes in its maturation with respect to shivering. Our
306 analysis suggests that high-altitude mice show a developmental delay in the expression of nearly
307 all pathways related to ATP generation by mitochondria, including glycolysis, TCA cycle, and
308 oxidative phosphorylation (Fig 4C). Many of the genes that contribute to functional enrichment
309 of metabolic pathways were related to the production of mitochondrial respiratory complex I,
310 including 31 of 35 *Nduf* genes, suggesting that downregulation of metabolic pathways may be
311 related to a delay in mitochondrial biogenesis. Indeed, the Gene Ontology terms “mitochondria
312 and mitochondrial part” were also significantly enriched (Table S3).

313 At least two transcription factors that are key regulators of cellular metabolism were
314 found in module M6 and were expressed at lower levels in highlanders (Fig. 4D-E). Estrogen-
315 related receptor alpha, *Esrra*, for example, directs the expression of genes involved in
316 mitochondrial biogenesis (Wu et al. 1999) and mitochondrial energy-producing pathways in
317 skeletal muscle (Huss et al. 2004). Genes in module M6 were also enriched for *Esrra* binding
318 sites (Table S3). Lowered expression of peroxisome proliferator activated receptor alpha (*Ppara*)
319 suggests that fatty acid oxidation may also be depressed in highlanders, since this gene is a major
320 regulator of fat metabolism capacity; functional terms related to fat oxidation however were not
321 enriched in this module (Supplemental Table S4). Indeed, (Robertson and McClelland 2019)
322 found that the β -oxidation enzyme β -hydroxyacyl-CoA dehydrogenase did not track changes in
323 muscle aerobic capacity of highlanders over these ages. These data provide evidence that the
324 developmental delay in thermogenic capacity is attributable to a delay in the expression of
325 pathways that facilitate mitochondrial development and muscle aerobic capacity and allow for
326 shivering thermogenesis.

327 *Evidence of natural selection*

328 We found that population-level divergence in expression of VO₂-associated
329 transcriptional modules exceeded neutral expectations set by genetic differentiation (measured as
330 genome-wide F_{ST}), suggesting that population differences in expression may be adaptive. We
331 expected that, if population-level divergence in module expression was adaptive, P_{ST} on module
332 eigengene would exceed the upper bounds of the confidence intervals around F_{ST}. At nearly
333 every value of c/h^2 , the lower 95% limit of P_{ST} for BAT modules B3 and B36 exceeded average
334 F_{ST} (Fig. 5). Moreover, at the null value of $c/h^2 = 1$ (Brommer 2011), P_{ST} was completely non-
335 overlapping with the upper 95% quantile of F_{ST}, indicating strong population-level
336 differentiation (Fig. 5 A-B). This finding is consistent with P_{ST} on cold-induced VO₂ values at
337 p10 (Supplemental Fig. S2), the time point of greatest population differentiation (Fig. 1A).
338 Although P_{ST} did not exceed F_{ST} for the skeletal muscle module M6 across all time points (p14-
339 p27), we did find it to exceed neutral expectations when restricted to p14 (Supplemental Fig. S2).
340 This is consistent with the finding that P_{ST} on thermogenic capacity also exceeds the upper 95%
341 of F_{ST} at p14 only (Supplemental Fig. S2). These data together suggest that the delay in the onset
342 of non-shivering and shivering thermogenesis, and their underlying regulatory control pathways,
343 may be the target of selection at high-altitude.

344 We note that approximately 25% of genes in candidate modules contain at least one SNP
345 above the PBS significance threshold (Table 3), and module B36 was significantly enriched for
346 PBS outliers. Because PBS is polarized by the use of a second lowland population (see
347 Methods), we can discern that these outlier loci are under positive selection at high-altitude
348 specifically. Together these results support the interpretation that differentiation in candidate
349 transcriptional modules, at least within BAT, is driven, in part, by natural selection.

350 *Summary and conclusions*

351 We found that a developmental delay in the onset of independent thermoregulatory
352 ability in deer mice native to high altitude is rooted in a delay in the expression of gene
353 regulatory networks that contribute to sympathetic innervation of BAT that permits the response
354 to cold, and in pathways that contribute to aerobic metabolism capabilities of both thermo-
355 effector organs. Phenotypic divergence analysis suggests that both thermogenic delays and
356 associated shifts in gene expression in BAT are driven by natural selection. An overabundance of
357 genes exhibiting signatures of natural selection in BAT module B36 suggests that developmental
358 changes to genes in this module may be particularly important in high-altitude adaptation. Our

359 results demonstrate that changes to the developmental trajectory of thermoregulation at high-
360 altitude are the result of regulatory delays in the development of thermo-effector organs, which
361 limit the ability of high-altitude mice to respond to cold and mount a thermoregulatory response.
362 Evidence presented here is consistent with the hypothesis that the developmental delay in
363 thermogenesis at high altitude is attributable to an adaptive energetic trade-off (Robertson et al.
364 2019; Robertson and McClelland 2019). Although the exact cause is unknown, the combination
365 of chronic cold and low O₂ availability at high-altitude, coupled with differences in litter size that
366 should affect competition among siblings for limited resources, may contribute to the altered
367 energetic conditions that drive this trade-off. Whatever the ultimate cause, our results suggest
368 that high-altitude pups may benefit from allocating limited resources away from active
369 thermoregulation, towards other developmental functions such as growth. Because high-altitude
370 adult deer mice show the opposite pattern (*i.e.*, consistently higher thermogenic capacities,
371 Cheviron et al. 2013), our combined results suggest that selection pressures at high altitude can
372 have very different effects on the same physiological system depending on age. Future work on
373 the selective drivers and energetic benefits of this putatively adaptive energy allocation strategy
374 will shed further light on adaptive modification of developmental processes to meet the
375 challenge of selection pressures that vary in strength and magnitude across ontogeny.

376 **Materials and Methods**

377 *Animals and Experimental Procedures*

378 Deer mice used in this study were the second-generation lab-reared descendants of two
379 wild populations of *Peromyscus maniculatus* (Robertson et al. 2019; Robertson and McClelland
380 2019) raised under common garden conditions (24°C, 80 m a.s.l.) at McMaster University of
381 Ontario, Canada. Individuals from the high-altitude population were trapped at the summit of
382 Mount Evans in Clear Creek County CO, USA (4,350 m a.s.l.) and the low-altitude population
383 from Nine-mile prairie, NE, USA (320 m a.s.l.). Altricial rodents, such as deer mice, usually
384 develop non-shivering thermogenesis (NST) prior to shivering due to the maturation of BAT
385 which precedes the maturation of skeletal muscle. *P. maniculatus* are only capable of NST after
386 the first 10 days postpartum (Robertson et al. 2019). In contrast, shivering develops between two
387 and four weeks after birth (Robertson and McClelland 2019). We sampled BAT and skeletal
388 muscle (specifically, the gastrocnemius) over these two periods. Briefly, pups were removed
389 from the nest, then euthanized with an overdose of isoflurane followed by cervical dislocation. In

390 pups ages 0, 2, 4, 6, 8 and 10 days postpartum the intrascapular depot of BAT was blunt
391 dissected and cleaned of white adipose tissue. In pups aged 14, 21, and 27 days postpartum the
392 gastrocnemius muscle of the lower hindlimb was bluntly dissected. Tissues were flash frozen and
393 stored at -80°C.

394 We re-analyzed cold-induced O₂ consumption rate (VO₂) data from Robertson et al.
395 (2019) and Robertson and McClelland (2019) for low- and high-altitude native *P. maniculatus*
396 only, omitting the low altitude congeneric, *P. leucopus*. Briefly, for pups aged 0-10 days
397 postpartum cold-induced VO₂ was assessed in response to a mild cold stress (10 minutes at
398 24°C). Pups were compared to a control litter mate who was maintained at 30°C for the same
399 duration of trial in order to control for handling stress. For pups aged 14-27 days, maximum
400 cold-induced VO₂ (hereafter, thermogenic capacity) was measured using previously established
401 methods for adult deer mice (Cheviron et al. 2012). VO₂ max was induced by exposing pups to -
402 4°C in heliox air (21% O₂ with He). All VO₂ measurements were divided by mass to obtain
403 mass-specific metabolic rates.

404 ***Transcriptome analyses***

405 We conducted high-throughput sequencing of RNA of BAT and gastrocnemius skeletal
406 muscle in order to explore the mechanisms that underlie developmental delays in thermogenesis.
407 Sample size varied from $n=1-7$ for each population, age, and tissue (see Table S1 in online
408 supplementary material for full list). We assayed gene expression using TagSeq, a 3' tag-based
409 sequencing method following Lohman et al. (2016). First, we extracted RNA from <25 mg of
410 tissue using TRI Reagent (Sigma-Aldrich), then assessed RNA quality using TapeStation
411 (Agilent Technologies; RIN > 7). The Genome Sequencing and Analysis Facility at the
412 University of Texas at Austin prepared TagSeq libraries, which were sequenced using Illumina
413 HiSeq 2500. We filtered raw reads for length, quality, and PCR duplicates following Lohman et
414 al. (2016) using scripts modified from those available online ([https://github.com/z0on/tag-](https://github.com/z0on/tag-based_RNAseq)
415 [based_RNAseq](https://github.com/z0on/tag-based_RNAseq)). Using the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), we cleaned
416 and trimmed raw reads, which resulted in 3.2 million reads per individual. Filtered reads were
417 then mapped to the *P. maniculatus* genome (NCBI GCA_000500345.1 Pman_1.0) using *bwa*
418 *mem* (Li and Durbin 2010). We used *featureCounts* (Liao et al. 2014) to generate a table of
419 transcript abundances. Since genes with low read counts are subject to measurement error
420 (Robinson and Smyth 2007), we excluded those with less than an average of 10 reads per

421 individual. We retained a total of 11,192 and 11,104 genes after filtering for BAT and skeletal
422 muscle transcriptomes, respectively. Five outlier samples (one BAT, four skeletal muscle) were
423 removed following visual inspection of multi-dimensional scaling plots using *plotMDS* in *edgeR*
424 following Chen et al. (2019).

425 We assessed overall patterns of gene expression among BAT and skeletal muscle
426 transcripts using Principal Components Analysis (PCA), while weighted gene co-expression
427 network analysis (WGCNA v. 1.41-1; Langfelder and Horvath 2008) was used to identify
428 potential regulatory mechanisms that underlie variation in thermogenesis across developmental
429 time. PCA and WGCNA analyses were conducted separately on BAT and skeletal muscle
430 samples in R v. 2.5.2 (R Core Team). WGCNA identified clusters of genes with highly
431 correlated expression profiles (hereafter, modules). This approach was successfully implemented
432 in recent studies that aimed to relate gene expression with higher-level phenotypes (Plachetzki et
433 al. 2014; Velotta et al. 2016; Velotta et al. 2018). Prior to performing WGCNA, we normalized
434 raw read counts by library size and log-transformed them using the functions *calcNormFactors*
435 and *cpm*, respectively, from the R package *edgeR* (Robinson et al. 2010). Module detection was
436 performed using the *blockwiseModules* function in WGCNA with *networkType* set to “signed”
437 (Langfelder and Horvath 2008). Briefly, Pearson correlations of transcript abundance data were
438 calculated between pairs of genes, after which an adjacency matrix was computed by raising the
439 correlation matrix to a soft thresholding power of $\beta = 6$. Soft thresholding power β is chosen to
440 achieve an approximately scale free topology, an approach that favors strong correlations (Zhang
441 and Horvath 2005). We chose a β since it represents the value for which improvement of scale
442 free topology model fit begins to decrease with increasing thresholding power. A topological
443 overlap measure was computed from the resulting adjacency matrix for each gene pair.
444 Topologically-based dissimilarity was then calculated and used as input for average linkage
445 hierarchical clustering in the creation of cluster dendrograms for both left and right ventricles.
446 Modules were identified as branches of the resulting cluster tree using the dynamic tree-cutting
447 method (Langfelder and Horvath 2008). We assigned modules a unique identification according
448 to tissue (B0-B37 for brown adipose tissue; M0-M7 for skeletal muscle). Genes that could not be
449 clustered into a module were given the designation B0 and M0 for BAT and skeletal muscle,
450 respectively.

451 Once modules were defined, we used a multi-step process to associate expression among
452 individuals with variation in VO₂. First, we summarized module expression using principal
453 components analysis (PCA) of gene expression profiles (*blockwiseModules* in WGCNA);
454 because genes within modules are highly correlated by definition, the first principal component
455 (referred to as the module eigengene value) was used to represent module expression (Langfelder
456 and Horvath 2008). We used module eigengene values to test for associations between module
457 expression and VO₂ for each module (Pearson correlation; *cor* function in WGCNA) in the BAT
458 network. *P*-values for the correlation were determined by a Student's asymptotic test
459 (*corPvalueStudent* in WGCNA). For skeletal muscle, association tests were conducted on
460 population-age means since VO₂ measurements do not exist for the individuals sequenced.
461 Among phenotype-associated modules, we conducted analysis of variance (ANOVA) on rank-
462 transformed module eigengene values in order to test for the effects of population, age, and their
463 interaction on module expression. *P*-values from association tests and ANOVAs were corrected
464 for multiple testing using the false-discovery rate method.

465 We performed functional enrichment analysis on all modules exhibiting either a
466 statistical association with VO₂, or a significant effect in ANOVA, or both, using the R package
467 *gProfilerR* (Reimand et al. 2016). *Peromyscus maniculatus* gene names were converted to *Mus*
468 *musculus* gene names using a custom script (Online supplementary material). We corrected for
469 multiple testing using *gProfilerR*'s native g:SCS algorithm. We used the list of genes from
470 filtered BAT and gastroc transcriptomes as a custom background list in all enrichment analyses.
471 We searched for enrichment among terms from Gene Ontology (GO; The Gene Ontology
472 Consortium 2019), KEGG (Kanehisa and Goto 2000), Reactome (Fabregat et al. 2018), and
473 WikiPathways (Slenter et al. 2018) databases, as well as transcription factor binding sites from
474 the TRANSFAC database (Matys et al. 2006).

475 *Tests of phenotypic and genetic divergence*

476 We used two complementary approaches to assess phenotypic and genetic divergence to
477 test for signatures of natural selection in VO₂-associated modules. To assess phenotypic
478 divergence, we calculated P_{ST} on module eigengene values following Brommer (2011) using Eq.
479 (1),

$$480 \quad P_{ST} = \frac{\frac{c}{h^2}\sigma_B^2}{\frac{c}{h^2}\sigma_B^2 + 2\sigma_W^2} \quad (1)$$

481 where σ_B^2 denotes between-population phenotypic variance, σ_W^2 denotes within-population
482 phenotypic variance, h^2 the narrow-sense heritability (the proportion of phenotypic variance that
483 is due to additive genetic effects), and c a scalar representing the total phenotypic variance due to
484 additive genetic effects across populations. Within- and between-population variances were
485 estimated from ANOVA, where module eigengene value were response variables, and postnatal
486 day, population, and family nested within population were included as main effects. Within-
487 population variance (σ_W^2) was calculated as the residual mean squares (MS) from the ANOVA
488 model. Between-population variance was estimated after Antoniazza et al (2010) following Eq.
489 (2),

$$490 \quad \sigma_B^2 = \frac{MS_B - MS_W}{n_0} \quad (2)$$

491 where MS_B and MS_W are the population effect and the residual mean square variances from the
492 ANOVA model, respectively, and n_0 a weighted average of sample size based on Sokal and
493 Rohlf (1995) following Eq. (3).

$$494 \quad n_0 = \frac{(n_1 + n_2) - (n_1^2 + n_2^2)}{n_1 + n_2} \quad (3)$$

495 where n_1 and n_2 are the sample sizes of population one and two, respectively. The ratio c/h^2 was
496 unknown and could not be readily estimated using our experimental design. As such, we
497 calculated P_{ST} across a range of values of c/h^2 from 0 (no heritability) to 2, which is where the
498 relationship between c/h^2 and P_{ST} begins to reach asymptote (Brommer 2011; Fig. 5). We
499 calculated 95% confidence intervals for each value of P_{ST} calculated across values of c/h^2
500 ranging from 0-2 at 0.05 level increments (total of 40 P_{ST} estimates).

501 P_{ST} estimates were compared to neutral divergence expectations set by between-
502 population differentiation. We calculated pairwise genetic differentiation between the lowland
503 and highland populations using F_{ST} (estimated with Weir's Theta; Weir and Cockerham 1984)
504 and previously sequenced exome data (Schweizer, Velotta, et al. 2019). Briefly, F_{ST} was
505 calculated for approximately 5 million high-quality bi-allelic single nucleotide polymorphisms
506 (SNPs) using the '--weir-fst-pop' flag within vcftools v0.1.16 (Danecek et al. 2011). We
507 compared P_{ST} of VO_2 -associated modules to that of "Module 0," which contains the suite of
508 genes that could not be reliably clustered (B0 in BAT; M0 in skeletal muscle). The null
509 expectation is that P_{ST} should not exceed the neutral expectation set by F_{ST} at any value of c/h^2 .
510 We expected that, if natural selection has shaped shifts in gene expression, then P_{ST} would

511 exceed F_{ST} in VO_2 -associated modules, but not in Modules B0 or M0 (which serve as a measure
512 of background levels of expression differentiation). We calculated P_{ST} on cold-induced VO_2 (p0-
513 p10) and thermogenic capacity (p14-p27) as above in order to determine whether these higher
514 level phenotypic traits were also the targets of natural selection.

515 Finally, we took a population genomic approach to assess whether genes in VO_2 -
516 associated transcriptional modules exhibit signatures of positive natural selection at high-
517 altitude. To do this, we re-analyzed Population Branch Statistic (PBS; Yi et al. 2010) data from
518 (Schweizer, Velotta, et al. 2019). Here, PBS identifies loci that exhibit extreme allele frequency
519 differences in highland (Mt. Evans) relative to lowland (Lincoln and Merced, CA) populations.
520 We previously calculated PBS for approximately 5 million exome-wide bi-allelic SNPs, then
521 calculated the demographically-corrected significance of PBS values using a simulated
522 distribution of 500,000 SNPs (see Schweizer, Velotta, et al. 2019 for simulation details). We first
523 determined significant outlier SNPs with a PBS value located above the 99.9% quantile, then
524 identified those SNPs located within genes belonging to VO_2 -associated modules using
525 Ensembl's Variant Effect Predictor (McLaren et al. 2010) with the *P. maniculatus* reference
526 genome annotation data set. We used Fisher's Exact Tests to determine whether thermogenesis-
527 associated modules were statistically enriched for loci that exceeded the 99.9% threshold.

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715 1128.xml

Table 1. Brown adipose tissue modules significantly correlated with cold-induced VO₂. Full association test results are presented in Supplementary Table S3. Effect of population (highland vs. lowland), age (postnatal day p0-p10), or their interaction from ANOVA models on rank-transformed module expression values (module eigengene values). Final two columns present results of Pearson correlation between module expression and cold-induced VO₂. *P*-values from association tests and ANOVAs were corrected for multiple testing using the false-discovery rate method.

Module	Module size	Population		Age		Pop x Age		VO ₂ correlation	
		F _{1,30}	<i>P</i>	F _{1,30}	<i>P</i>	F _{1,30}	<i>P</i>	<i>r</i>	<i>P</i>
B2	1022	7.4	0.41	3.2	1.0	5.36	1.0	-0.49	0.02
B3	773	27.0	<0.001	20.8	<0.001	1.67	1.0	0.67	<0.001
B4	252	18.6	0.01	38.1	<0.001	0.43	1.0	-0.56	0.004
B11	99	162.1	<0.001	26.8	<0.001	0.04	1.0	-0.47	0.02
B12	605	21.5	<0.001	128.2	<0.001	4.90	1.0	-0.81	<0.001
B16	149	2.7	1.0	25.9	<0.001	0.06	1.0	-0.55	0.01
B32	267	0.1	1.0	32.9	<0.001	0.36	1.0	-0.52	0.01
B33	1073	0.7	1.0	53.9	<0.001	0.85	1.0	0.64	<0.001
B35	74	37.3	<0.001	0.9	1.0	0.02	1.0	-0.45	0.03
B36	708	26.5	<0.001	51.5	<0.001	1.52	1.0	0.58	<0.001

Table 2. Association-test and ANOVA results on skeletal muscle modules. Effect of population (highland vs. lowland), ontogenetic age (postnatal day p14-p27), or their interaction on rank-transformed module expression (module eigengene values) is presented. Final two columns present results of Pearson correlation between module expression and thermogenic capacity. Note that correlations were performed on population-age means since VO₂ measurements did not exist for the individuals sequenced. *P*-values from association tests and ANOVAs were corrected for multiple testing using the false-discovery rate method.

Module	Module size	Population		Age		Pop x Age		VO ₂ correlation	
		F _{1,22}	<i>P</i>	F _{1,22}	<i>P</i>	F _{1,22}	<i>P</i>	<i>r</i>	<i>P</i>
M1	92	3.4	0.6	15.0	0.006	2.4	1	0.18	0.99
M2	2654	1.0	1	150.5	<0.001	0.3	1	-0.94	0.02
M3	1052	11.9	0.02	1.8	1	2.3	1	-0.79	0.15
M4	288	97.8	<0.001	8.7	0.06	1.7	1	0.0002	0.99
M5	168	4.6	0.4	3.5	0.61	4.4	0.4	0.19	0.99
M6	2878	3.2	0.7	64.4	<0.001	0.1	1	0.98	0.004
M7	443	91.1	<0.001	7.2	0.11	1.0	1	0.07	0.99

Table 3. Number and proportion of genes in candidate modules containing outlier SNPs (PBS > 99.9% quantile of the simulated distribution). P-values obtained from Fisher's Exact Tests

Module	No. outlier genes	Total genes	% outlier genes	<i>p</i>-value
B3	184	773	23.8%	0.7
B36	196	708	27.7%	0.02
M6	631	2878	21.9%	1

Figure 1. The development of thermogenesis is delayed in highland (blue) related to lowland (orange) deer mice. (A) Cold (24°C)-induced O₂ consumption rate (VO₂) across postnatal day p2-p10. Values constitute the difference between cold-induced and normothermic VO₂, which controls for the stress of separating pups from their mothers at a young age. Values crossing zero indicates that pups are able to generate heat independently. (B) Thermogenic capacity (VO₂ max during exposure to 5°C in heliox) under normoxia is significantly lower in highlanders than lowlanders at p14. Values represent mean mass-corrected VO₂. *p<0.05; **p<0.01. Data from Robertson et al. 2019 (A) and Robertson and McClelland 2019 (B).

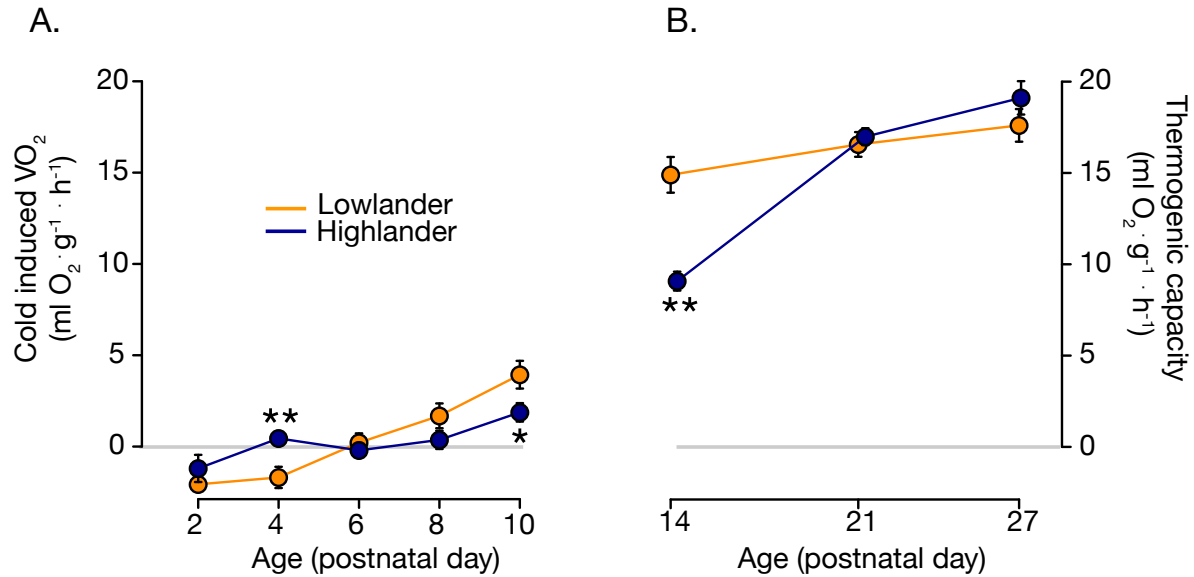


Figure 2. Principal components analysis (PCA) transcriptome expression data for thermo-effector organs brown adipose tissue and (A) skeletal muscle (B) in lowland (orange points) and highland (blue points) deer mice. Symbols represents ages from postnatal day p0-p10 for brown adipose tissue, and p14-p27 in skeletal muscle. Populations separated along PC1 in brown adipose tissue data and PC2 in skeletal muscle data. Grey circles are 95% confidence ellipses drawn around each population to highlight separation.

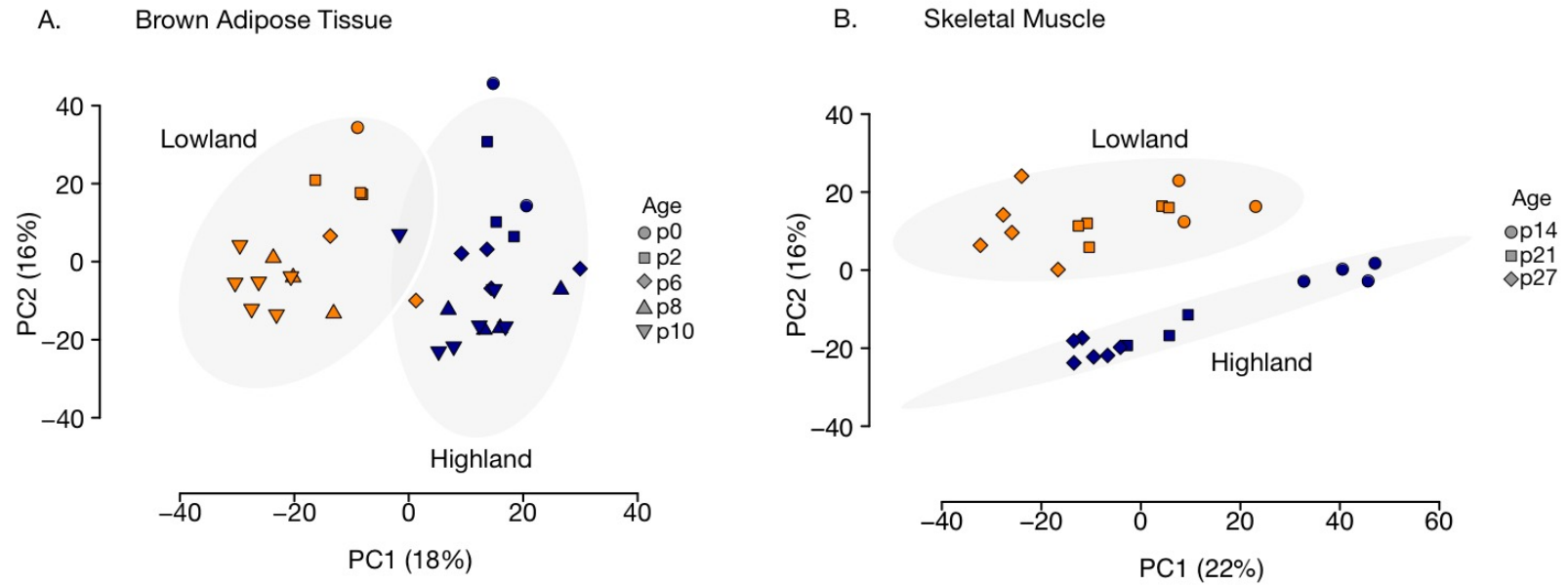


Figure 3. Two brown adipose tissue modules are associated with variation in thermogenesis in p0-p10 deer mouse pups. (A-B): panels show the significant positive association between module eigengene and cold-induced VO₂ (measure of thermogenesis). Eigengene values of modules B3 (C) and B36 (D) are significantly affected by age, and significantly differentiated between lowland (orange) and highland (blue) populations. We detected significant enrichment of KEGG pathways related to metabolism and fatty acid degradation in B3 (E), and VEGF signaling and axon guidance in B36, among others (F). Values in (E-F) are the negative log of the enrichment test *p*-value after correction for multiple testing using the g:SCS algorithm in *gProfilerR* (Reimend et al. 2016). Inset in (F) shows average expression (log counts per million; cpm) across age for *Vegfa* in lowlanders (orange) vs. highlanders (blue). *** *p*<0.001. See Table S3 for full functional enrichment analysis results.

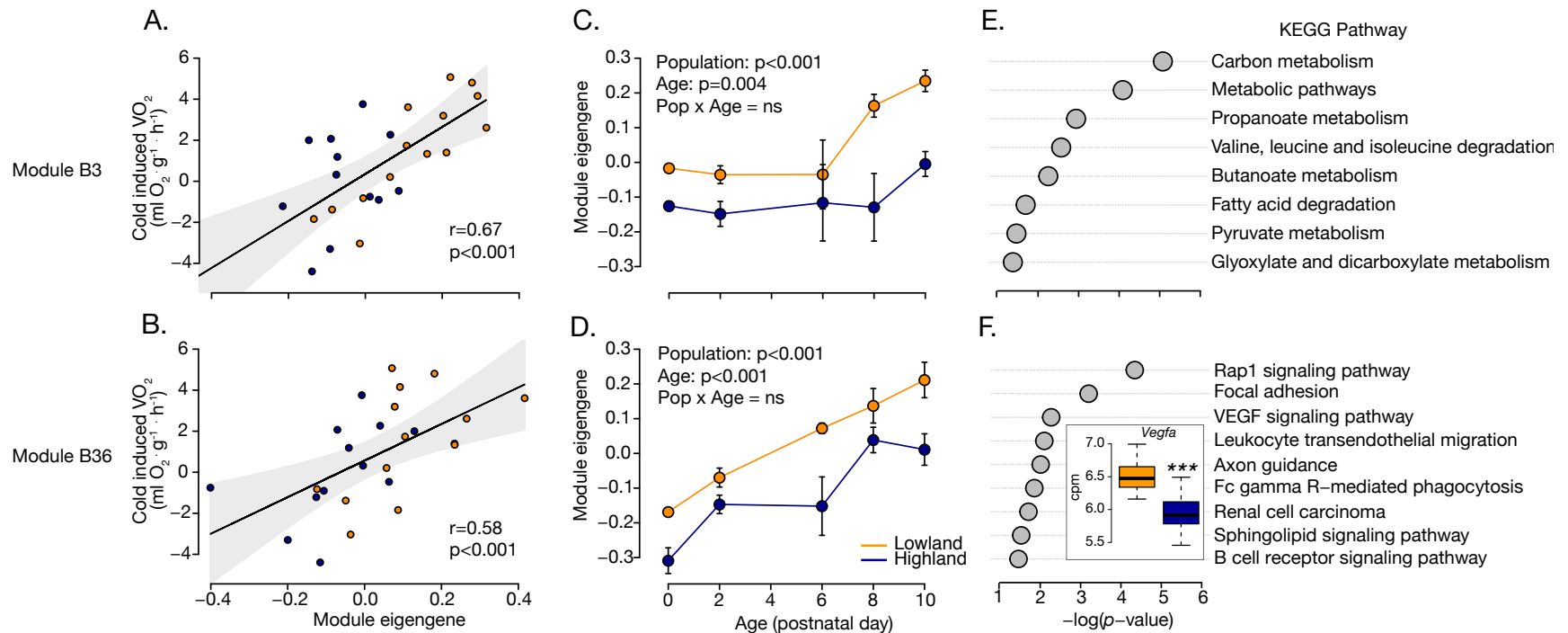


Figure 4. Skeletal muscle module M6 is significantly associated with thermogenic capacity in lowland (orange) and highland (blue) deer mice aged p14-p27. (A) Module expression is positively correlated with thermogenic capacity at the population/age level (individual level data not available). (B) Highland deer mice exhibit significantly lower module eigengene values at postnatal day p14 (** $p < 0.01$), and no differences in expression at p21 or p27. (C) Functional enrichment analysis reveals enrichment of KEGG pathways involved in aerobic metabolism and thermogenesis among others. Values are the negative log of the enrichment test p -value after correction for multiple testing using the g:SCS algorithm in *gProfilerR* (Reimend et al. 2016). Expression values (log counts per million; cpm) for the transcription factors *Esrra* (D) and *Ppara* (E) at p14 are lower for highlanders compared to lowlanders; both transcription factors are involved in the regulation of mitochondrial biogenesis and metabolic pathways.

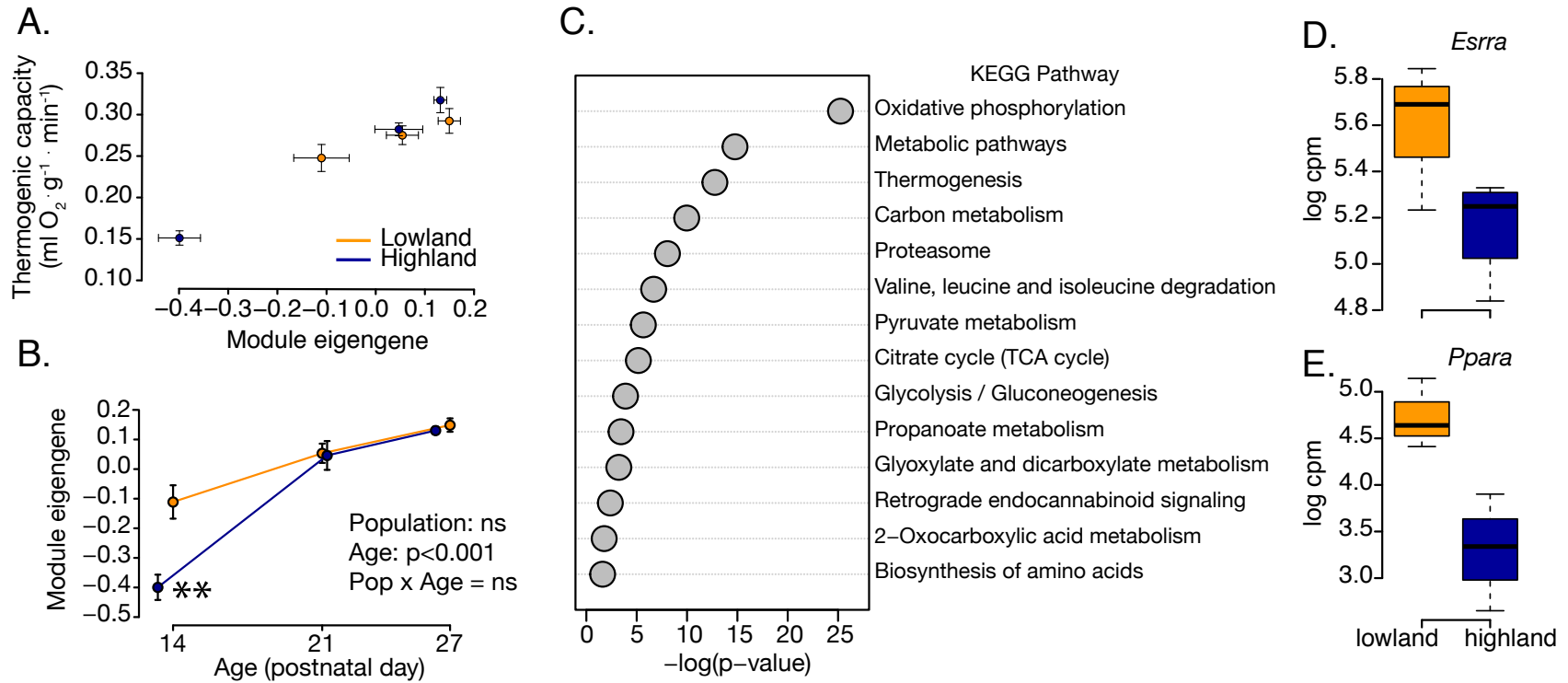
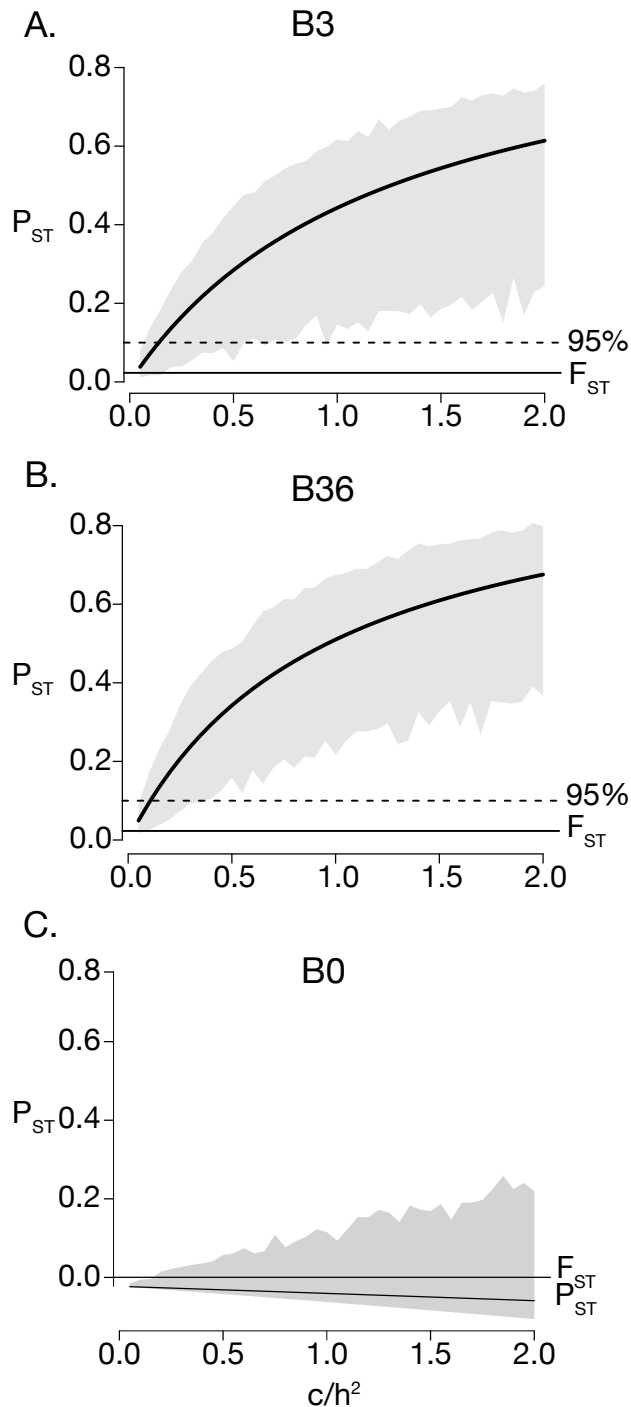


Figure 5. P_{ST} on module expression values for brown adipose tissue modules B0 (A), B3 (B), and B36 (C), which constitutes a suite of uncorrelated genes. P_{ST} values were calculated across a range of c/h^2 values from 0-2. Grey polygons represent 95% confidence limits on P_{ST} generated from bootstrapping. Average F_{ST} and the upper 95% quantile of F_{ST} are shown. For nearly every value of P_{ST} for modules B3 and B36, but not B0, the lower confidence limit of P_{ST} of module expression is non-overlapping with F_{ST} .



Supplementary Material

Table S1. Sample sizes for tag-seq analysis after outlier removal (see Methods).

	p0	p2	p6	p8	p10	p14	p21	p27
Lowland	2	4	4	4	6	3	3	5
Highland	1	2	2	3	6	4	5	6

Table S2. Table of all genes expressed in brown adipose tissue (BAT) and skeletal muscle and their module assignments using WGCNA. Modules were given color name identifiers by WGCNA, which were converted into numbered identifiers (B# for brown adipose tissue; M# for skeletal muscle). Gene names derived from the *Peromyscus maniculatus* genome Annotation Release 100.

Table S3. ANOVA and association analysis results for BAT modules. Table presents F-values and false-discovery rate corrected *p*-values for main effects of population, postnatal age, and their interaction from the ANOVA on rank-transformed module expression values. Pearson correlation coefficients and false-discovery rate corrected *p*-values for the association between VO₂ and module expression are also presented.

Table S4. Results of *gProfiler* functional enrichment analysis of phenotype-associated BAT and skeletal muscle WGCNA modules. Source represents whether terms were identified in the Gene Ontology (GO) database (BP: Biological Process, CC: Cellular Component, MF: Molecular Function), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Reactome pathways (REAC), Wikipathways (WP), or transcription factor binding sites identified in the TRANSFAC TFBS database (TF). ‘Adjusted_p_value’ is the corrected *p*-value for the enrichment analysis - we corrected for multiple testing using *gProfiler*’s native g:SCS algorithm. ‘Term_size’ represents the number of genes in each functional category term, while ‘query_size’ represents the number of searchable genes in each module. The column ‘intersections’ lists the gene query names associated with each term. Modules without reported terms had none significantly enriched at *p* > 0.05 after correction for multiple testing.

Figure S1. P_{ST} on module expression values for skeletal muscles modules M6 (A), and M0, which constitutes the suite of uncorrelated genes. P_{ST} values were calculated across a range of c/h^2 values from 0-2. Grey polygons represent 95% confidence limits on P_{ST} generated from bootstrapping. Average F_{ST} and the upper 95% quantile of F_{ST} are shown.

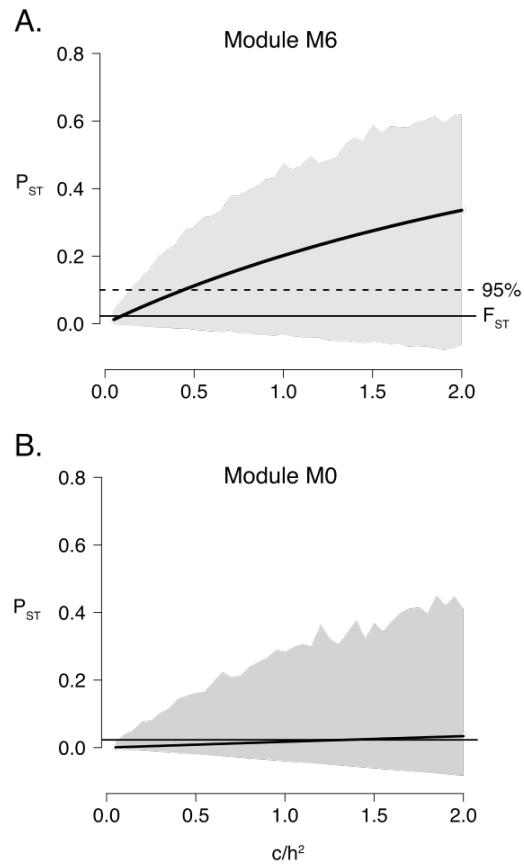


Figure S2. P_{ST} on cold-induced VO_2 (A) and thermogenic capacity data (D) at the time point of greatest population differentiation (p10 and p14, respectively). Also plotted is P_{ST} on candidate module eigengene values B3 (B), B36 (C), and M6 (E) for at the same time points. P_{ST} values were calculated across a range of c/h^2 values from 0-2. Grey polygons represent 95% confidence limits on P_{ST} generated from bootstrapping. Average F_{ST} and the upper 95% quantile of F_{ST} are shown.

