

RESEARCH ARTICLE

Hibernating above the permafrost: effects of ambient temperature and season on expression of metabolic genes in liver and brown adipose tissue of arctic ground squirrels

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SUMMARY

Hibernating arctic ground squirrels (*Urocitellus parryii*), overwintering in frozen soils, maintain large gradients between ambient temperature (T_a) and body temperature (T_b) by substantially increasing metabolic rate during torpor while maintaining a subzero T_b . We used quantitative reverse-transcription PCR (qRT-PCR) to determine how the expression of 56 metabolic genes was affected by season (active in summer vs hibernating), metabolic load during torpor (imposed by differences in T_a : +2 vs -10°C) and hibernation state (torpid vs after arousal). Compared with active ground squirrels sampled in summer, liver from hibernators showed increased expression of genes associated with fatty acid catabolism (*CPT1A*, *FABP1* and *ACAT1*), ketogenesis (*HMGCS2*) and gluconeogenesis (*PCK1*) and decreased expression of genes associated with fatty acid synthesis (*ACACB*, *SCD* and *ELOVL6*), amino acid metabolism, the urea cycle (*PAH*, *BCKDHA* and *OTC*), glycolysis (*PDK1* and *PFKM*) and lipid metabolism (*ACAT2*). Stage of hibernation (torpid vs aroused) had a much smaller effect, with only one gene associated with glycogen synthesis (*GSY1*) in liver showing consistent differences in expression levels between temperature treatments. Despite the more than eightfold increase in energetic demand associated with defending T_b during torpor at a T_a of -10 vs $+2^\circ\text{C}$, transcript levels in liver and brown adipose tissue differed little. Our results are inconsistent with a hypothesized switch to use of non-lipid fuels when ambient temperatures drop below freezing.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/8/1300/DC1>

Key words: arctic ground squirrel, fuel substrate, metabolism, real-time PCR, thermogenesis, torpor, hibernation.

INTRODUCTION

Hibernation in mammals is characterized by a suite of interconnected morphological, physiological, molecular and behavioral changes that enable individuals to persist in environments with seasonal or unpredictable shortages in energy supply. During prolonged winters in the Arctic, reduced food availability is accompanied by a lack of daylight and frigid temperatures. In these conditions, the arctic ground squirrel (*Urocitellus parryii*) may exhibit the most extreme hibernation physiology known: it is capable of supercooling its core body temperature (T_b) to -2.9°C [the lowest T_b adopted by any mammal (Barnes, 1989)] and surviving winters as long as 250 days sequestered in frozen hibernacula. During their hibernation they subsist solely on endogenous reserves (Buck and Barnes, 1999a; Buck and Barnes, 1999b) and are heterothermic, alternating between several week-long torpor bouts and arousal episodes of less than 1 day (Fig. 1).

Through metabolic inhibition and temperature or Q_{10} effects on enzyme kinetics, arctic ground squirrels suppress metabolic rates during torpor to $0.01\text{ ml O}_2\text{ g}^{-1}\text{ h}^{-1}$ or 2% of basal levels for up to 24 days at a time (Karpovich et al., 2009; Buck and Barnes, 2000), levels that are comparable to the minimum rates of metabolism observed during torpor in all hibernators (reviewed in Geiser and Ruf, 1995). However, in their high latitude habitats, arctic ground

squirrels experience natural thermal conditions that are far more severe in winter than conditions experienced by temperate or alpine-dwelling hibernators. Temperatures within their hibernacula average -8.9°C over seven winter months (October–April), with minima of -23.4°C (Buck and Barnes, 1999b). With the exception of the Alaska marmot (Lee et al., 2009), other small mammalian hibernators have rarely, if ever, been shown to experience hibernacula temperatures of $<0^\circ\text{C}$, and they typically do not support a significant thermal gradient between T_b and ambient temperature (T_a) (Kenagy et al., 1989; Young, 1990; Michener, 1992; Ferron, 1996). Thus, even with the thermal advantage conferred by reaching a subzero T_b during torpor, arctic ground squirrels must be continuously thermogenic throughout the majority of the hibernation season as they sustain increasing gradients between T_b and T_a . This is energetically costly: torpid metabolic rate is more than eightfold higher in animals hibernating at -10°C compared with those at 2°C (Buck and Barnes, 2000). Maintenance of a stable body temperature during torpor at subzero ambient temperatures is thought to be achieved almost exclusively through non-shivering thermogenesis within brown adipose tissue (BAT), using lipid as the fuel substrate. However, Buck and Barnes observed a concomitant increase in respiratory quotient (RQ) with decreasing T_a below 0°C , indicative of a shift from exclusive catabolism of lipids when thermoneutral

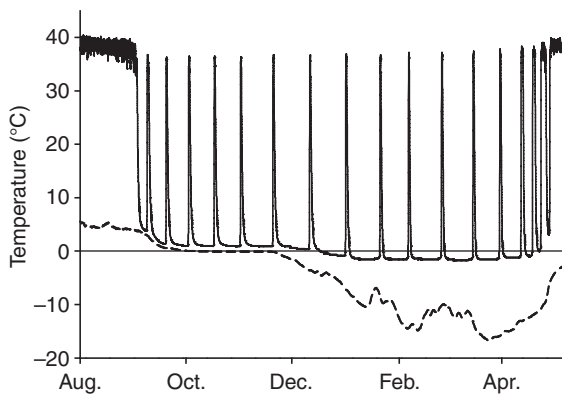


Fig. 1. Plot of core body temperature (T_b ; solid line) and nearby soil temperature (dashed line) vs time for a free-living arctic ground squirrel overwintering near Toolik Lake, Alaska (68°N , 149°W). The hibernation season is composed of extended bouts of multiday torpor interrupted by periodic arousals during which squirrels return to euthermic T_b for ~15 h. The large difference between torpid T_b and soil temperature is maintained through non-shivering thermogenesis.

($T_a=+2^\circ\text{C}$) to increased reliance on mixed fuel metabolism when animals are thermoregulating ($T_a<-3^\circ\text{C}$) (Buck and Barnes, 2000). We hypothesize that the change in RQ reflects an increasing use of glucose as the rate of thermogenesis increases (Buck and Barnes, 2000). During torpor, glucose is provided from blood and glycogen reserves, which then are replenished over arousal episodes (Galster and Morrison, 1975) through gluconeogenesis from glycerol and amino acid substrates. Catabolism of protein is supported by the observation that, although large lipid stores are accumulated during the summer active period and used throughout hibernation, free-living arctic ground squirrels also lose substantial amounts of lean tissue, as much as 26% (Buck and Barnes, 1999a).

Changes in the regulation of metabolism associated with mammalian hibernation has been the focus of intensive research for decades (reviewed in Gorer, 1930; Lyman and Chatfield, 1955; Geiser, 2004), and recently these efforts have been expanded to include changes in patterns of gene expression that underlie the hibernating phenotype (Williams et al., 2005; Yan et al., 2006; Yan et al., 2008). However, gene expression studies to date have focused on hibernation where ambient temperatures are $>0^\circ\text{C}$ and body temperatures differ only slightly from ambient conditions. Here we examine for the first time how patterns of gene expression are affected by changing ambient conditions in the hibernaculum. Because the previously observed increase in RQ with decreasing T_a suggests that low T_a causes a fundamental shift in metabolic fuel selection, we elected to use a target gene approach [real-time quantitative reverse-transcription PCR (qRT-PCR)] focused on genes associated with fatty acid metabolism, ketogenesis, amino acid metabolism, glycolysis and gluconeogenesis. We also examined the effects of season and stage of hibernation (torpid vs aroused) on variability in gene expression associated with T_a .

MATERIALS AND METHODS

Study subjects

Arctic ground squirrels (*Urocitellus parryi* Richardson 1825) were trapped in 2005–2009 on the north slope of Alaska near Toolik Lake (68°N , 149°W , elevation 809 m) and transported to the University of Alaska Fairbanks. Animals were individually housed initially at $18\pm 2^\circ\text{C}$ with a 16 h:8 h light:dark (16L:8D) photoperiod and provided with Mazuri Rodent Chow (PMI Nutrition, Henderson,

CO, USA) and water *ad libitum*, with supplements of sunflower seeds, carrots and apples. In late September, we transferred animals into environmental chambers held at either $+2^\circ\text{C}$ or -10°C with a photoperiod of 4 h:20 h light:dark. The 4 h of light per day was maintained to facilitate animal care. Animals were held in $17\times 9\times 8$ inch plastic or $19\times 12.5\times 8.25$ inch hanging metal cages and were provided with ample cotton material from which they constructed nests. Rodent chow, water and carrots (as a water source for animals at -10°C T_a) were provided until animals first entered torpor, after which all food was removed.

We conducted two experiments to determine how transcription of metabolic genes was affected by season, ambient temperature and hibernation state. A different set of squirrels was used in each experiment. Animals included in the experiments were all adults with the exception of a single juvenile in the $+2^\circ\text{C}$ hibernation group in the first experiment. A mixture of both sexes was used; low sample size, however, precluded us from testing for sex differences in gene expression. All squirrels sampled during hibernation had completed at least two full-length torpor bouts and none were sampled after February (i.e. >1 month of hibernation remained, based on typical hibernation patterns). Animals that were sampled during the post-reproductive period or during arousal (natural or induced) were deeply anesthetised using 5% isoflurane prior to killing by injection of sodium pentobarbital into the heart followed by decapitation. Torpid animals were killed by decapitation without anaesthesia. Liver and BAT samples were rapidly dissected and frozen in liquid nitrogen within 10 min and then stored at -80°C until total RNA was extracted at a later date.

Experiment 1

In the first experiment, we compared liver tissue from ground squirrels sampled during summer with that from squirrels hibernating in chambers at $+2$ and -10°C ($N=6$ per group). Squirrels sampled to represent the hibernation period were induced to arouse from torpor by placing them at room temperature (20°C) and inserting a thermocouple into the rectum. These squirrels were monitored during hibernation using the sawdust method, i.e. wood shavings were placed on the dorsal surface of the animals and inspected once daily to assess the occurrence of arousal episodes. Prior to inducing arousal, squirrels had been torpid for 7–12 days (mean \pm s.d.= 9.8 ± 1.5 days). Squirrels were killed and sampled 10 h after rectal temperature passed 30°C during rewarming. Ground squirrels sampled to represent the summer non-hibernation period were killed between 29 June and 14 July after they had spontaneously ended hibernation in April–May and had recently become post-reproductive. These animals were housed in chambers at $+2^\circ\text{C}$ with a 16L:8D photoperiod and were supplied with food and water.

Experiment 2

In the second experiment, we compared gene expression in liver and BAT from squirrels hibernating in $+2$ and -10°C environmental chambers during naturally occurring arousal episodes ($N=5$ per group) and during late torpor ($+2^\circ\text{C}$: $N=5$; -10°C : $N=6$). Following Yan et al. (Yan et al., 2008), we defined late torpor as $>80\%$ of the mean duration of the two prior torpor bouts; the number of days torpid ranged from 5 to 15 (10.1 ± 3.5 days). Squirrels sampled during late torpor were monitored using the sawdust method, as described in Experiment 1. Squirrels sampled during spontaneous arousal episodes had been implanted with temperature-sensitive radiotransmitters within their abdominal cavities (Long et al., 2007) in August before moving them into cold chambers. We monitored

torpor and arousal bouts in these animals using an automated data collection system (Data Sciences International, St Paul, MN, USA) that recorded core abdominal T_b every 10 min. Squirrels sampled during spontaneous arousals were killed 10 h after core T_b had reached 30°C, as indicated by radiotelemetry.

RNA isolation and reverse transcription

Total RNA was isolated from frozen tissues using an RNeasy mini kit (Qiagen Inc., Valencia, CA, USA). Liver samples were homogenized in RLT buffer and BAT samples in QIAzol reagent using a mini-bead beater (FastPrep-FP120, Qbiogene, Inc., Carlsbad, CA, USA) for 1 min (liver) or 30 s (BAT) at 4800 rpm. We applied RNase-free DNase (Qiagen) directly to the column to digest contaminant genomic DNA during RNA purification. The density of total RNA for each sample was determined using a NanoDrop spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA), and RNA quality was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was ≥ 9.2 for all liver samples and ≥ 8.6 for BAT samples. Thus, RNA quality was well above the minimum RIN value of 8 recommended for quantitative downstream analyses (Fleige and Pfaffl, 2006). Liver and BAT cDNA were synthesized from total RNA using a TaqMan[®] reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). For cDNA synthesis, 5.5 μl MgCl_2 (25 nmol l^{-1}), 2.5 μl 10 \times RT buffer, 5 μl dNTP mix (10 mmol l^{-1}), 1.25 μl Oligo d(T)₁₆, 0.5 μl RNase inhibitor and 0.62 μl reverse transcriptase were added to 500 ng of total RNA in 9.63 μl H₂O for a total volume of 25 μl . The following thermal profile was used for the reverse transcription reaction: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The synthesized cDNA was 10 \times diluted using RNase-free water.

qRT-PCR

For the majority of genes tested (37 of 56), we designed primers based on arctic ground squirrel EST sequences obtained from the EST sequencing project at University of Alaska Fairbanks using Primer3 software (<http://primer3.sourceforge.net>). For the remaining genes, we used primers previously developed based on the ground squirrel sequences pooled from arctic, golden-mantled [*Callospermophilus lateralis* (Say 1823)] and 13-lined [*ICTIDOMYS TRIDECIMLINEATUS* (Mitchell 1821)] ground squirrels (Yan et al., 2008). The sequences of primer pairs for each gene along with the gene name are listed in supplementary material Table S1. We performed real-time qRT-PCR in triplicate using Power SYBR Green Master Mix on an ABI-7900 HT system (Applied Biosystems). We used 4 μl of diluted cDNA solution in each 20 μl real-time qRT-PCR reaction. Cycle parameters were 50°C for 2 min of incubation, 95°C for 10 min of *Taq* activation, 40 cycles of 95°C for 15 s and 60°C for 1 min followed by a disassociation curve to verify amplification of a single product. Controls with no template were taken to exclude contamination, and controls with no reverse transcriptase but all other components were taken to exclude false amplification from genomic DNA. For each primer set, we created a standard curve of four 10-fold dilutions of a pooled cDNA sample. All reactions showed an efficiency of no less than 88% and standard curve correlation coefficients were 0.98 or higher.

For each set of qRT-PCR experiments, we selected an endogenous housekeeping gene for normalization from 10 potential reference genes. We selected the most stable gene identified by GeNorm (Vandesompele et al., 2002) and NormFinder software (Anderson et al., 2004) that did not show a significant difference in expression among groups. In the first and second experiments, the selected

genes for normalization of expression in liver tissue were *RPS3* and *HPRT1*, respectively. In the second experiment, we used *YWHAZ* for normalization in BAT samples. Two of these genes (*HPRT1* and *YWHAZ*) have previously been identified as stable housekeeping genes in hibernating ground squirrels (Otis et al., 2010). For each experiment, the relative quantification of the target transcript level in each sample was related to a control group (non-hibernating squirrels in the first experiment and torpid squirrels at -10°C T_a in the second experiment) using the method described by Pfaffl (Pfaffl, 2001), which takes into account PCR efficiencies of target and reference genes.

Statistical analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). We report means \pm s.d. in the text and supplemental tables and means \pm s.e.m. in figures. For each animal equipped with a temperature transmitter, we calculated the mean abdominal temperature during the last torpid bout and tested for differences between groups hibernating at -10 and $+2^\circ\text{C}$ using one-way ANOVA. To ensure that we were not including temperature data collected during the initiation of arousals, we excluded any temperature measurements taken within 48 h of a core T_b of 4°C.

We tested for differences in gene expression between groups using Welch's ANOVA (allowing for unequal variance) and adjusted *P*-values using the false discovery rate (FDR) method of Benjamin and Hochberg (Benjamin and Hochberg, 1995). In the first experiment, we excluded one squirrel in the post-reproductive group from all analyses because both raw cycle threshold (C_t) values and normalized gene expression values were outliers for a large proportion of genes. To determine which groups differed from one another we followed significant ANOVAs (FDR-adjusted $P < 0.05$) with *post hoc* Dunnett's T3 tests, which also allow for unequal variance. For each tissue in each experiment we also performed a principal components analysis (PCA) on normalized gene expression values. PCA is a mathematical algorithm that provides a means of reducing the dimensionality of the data set so that the majority of the variance is represented in a few uncorrelated principal components. This approach has been effective in identifying and displaying differences in expression across a multitude of genes in previous gene expression studies (reviewed in Ringnér, 2008). We tested for differences in principal components between groups using ANOVA and FDR-adjusted *P*-values followed by Tukey's honestly significant difference (HSD) tests.

RESULTS

Body temperature

The mean abdominal temperature of torpid arctic ground squirrels exposed to ambient temperatures of -10°C was $-0.7 \pm 0.2^\circ\text{C}$, significantly lower than that of squirrels hibernating at $+2^\circ\text{C}$ ($2.2 \pm 0.3^\circ\text{C}$, $F_{1,8} = 316.3$, $P < 0.0001$). The gradient between abdominal and ambient temperatures was higher in squirrels hibernating at -10°C ($=9.3^\circ\text{C}$) compared with squirrels at $+2^\circ\text{C}$ ($=0.2^\circ\text{C}$), indicating that animals at -10°C were generating more heat on a steady-state basis.

Metabolic gene expression

In the first experiment, we compared gene transcript levels in liver between post-reproductive summer active squirrels with that of two groups of hibernating squirrels sampled during induced arousals and housed either at $+2$ or -10°C . We detected significant differences (FDR-adjusted $P < 0.05$) in expression among groups for 15 of 56

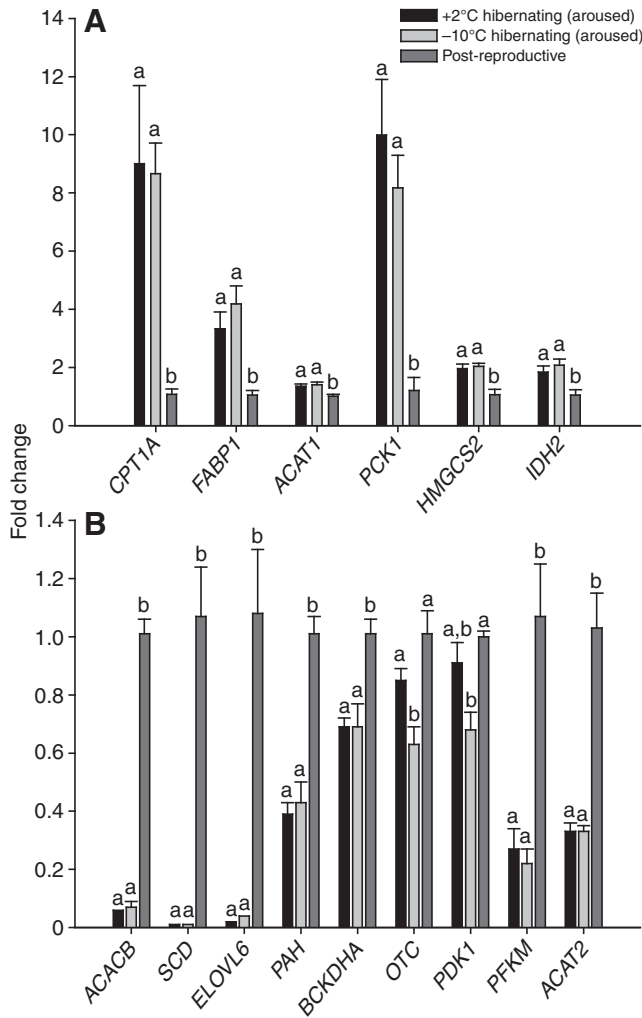


Fig. 2. Fold change in gene expression in liver tissue for (A) six genes significantly overexpressed (FDR-adjusted $P < 0.05$) and (B) nine genes significantly underexpressed in hibernating arctic ground squirrels relative to post-reproductive individuals. Different letters indicate significant differences between groups (Dunnett's T3, $P < 0.05$) for each gene. A significant difference in gene expression between +2 and -10°C hibernators was only found for *OTC*. Data are means \pm s.e.m.

genes tested (27%; Fig. 2). Complete results of ANOVA tests for this experiment are given in supplementary material Table S2. Six genes were significantly overexpressed in both groups of hibernators relative to post-reproductive summer individuals: three genes involved in fatty acid catabolism (*CPT1A*, *FABP1* and *ACAT1*), one associated with ketogenesis (*HMGCS2*), one gluconeogenic gene (*PCK1*) and one gene involved in the tricarboxylic acid (TCA) cycle (*IDH2*). Seven genes were underexpressed in both groups of hibernators relative to summer animals and two genes were underexpressed in the group of hibernators housed at -10°C relative to the summer group. Genes underexpressed in hibernators were associated with fatty acid synthesis (*ACACB*, *SCD* and *ELOVL6*), amino acid metabolism (*PAH*, *BCKDHA* and *OTC*), glycolysis (*PDK1* and *PFKM*) and lipid metabolism (*ACAT2*). Transcript levels of only one gene differed between squirrels hibernating at $+2^{\circ}\text{C}$ and those held at -10°C : *OTC* was slightly overexpressed (1.3-fold higher) in the $+2^{\circ}\text{C}$ group relative to the -10°C group. We also

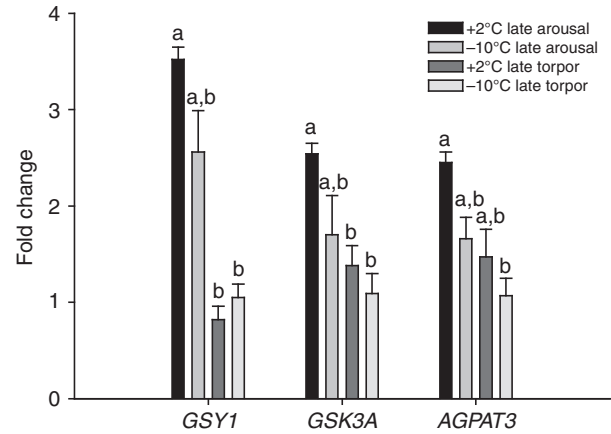


Fig. 3. Fold change in gene expression in liver tissue for three genes exhibiting differential expression within hibernating arctic ground squirrels. Different letters indicate significant differences between groups (Dunnett's T3, $P < 0.05$). Data are means \pm s.e.m.

performed a PCA on the normalized expression levels for the 56 genes. The first, second and third principal components (PC1, PC2 and PC3) accounted for 30.6, 22.4 and 8.5% of the total variation, respectively. PC1 was significantly affected by group ($F_{2,14}=28.5$, FDR-adjusted $P=0.003$, $R^2=0.80$); *post hoc* Tukey's tests revealed that both hibernator groups differed from the post-reproductive group but not from one another. PC2 was not affected by group ($F_{2,14}=1.1$, $P=0.37$) whereas PC3 was ($F_{2,14}=7.3$, $P=0.004$, $R^2=0.55$). *Post hoc* Tukey's tests indicated that $+2^{\circ}\text{C}$ hibernators had a different mean PC3 value than -10°C hibernators but neither group differed from post-reproductive squirrels. Thus, metabolic gene expression in liver differed significantly between animals hibernating at different temperatures, although our analyses indicate that T_a during hibernation explained little ($<5\%$) of the total variation in transcript levels.

In the second experiment, we compared gene transcript levels in ground squirrels hibernating at $+2$ and -10°C during late torpor and late in the euthermic phase of a spontaneous arousal episode. For liver samples, real-time PCR revealed significant differences in transcript levels for three of the 55 genes tested (6%, Fig. 3). Supplementary material Table S3 summarizes the results of ANOVA tests for liver tissue in Experiment 2. *Post hoc* Dunnett's T3 tests indicated no significant differences between the $+2$ and -10°C groups within a hibernation stage. Two genes, both of which are associated with glycogen synthesis (*GSY1* and *GSK3A*), were significantly overexpressed after arousal *versus* in torpor at $+2^{\circ}\text{C}$, but differences between after arousal and late torpor were not significant at -10°C . The third gene that was differentially expressed (*AGPAT3*) is involved in FA synthesis and exhibited higher levels in aroused squirrels at $+2^{\circ}\text{C}$ compared with torpid squirrels at -10°C . The first, second and third PCs from a PCA on normalized gene expression levels accounted for 40.9, 14.5 and 11.8% of the total variation, respectively. Although PC1 was not significantly affected by group ($F_{3,17}=2.32$, $P=0.11$), both PC2 ($F_{3,17}=6.95$, $P=0.009$, $R^2=0.55$) and PC3 ($F_{3,16}=5.96$, $P=0.009$, $R^2=0.51$) were. *Post hoc* Tukey's tests revealed the mean PC2 values differed between temperature groups but not between hibernation states, whereas PC3 values were lower in the arousal group from the $+2^{\circ}\text{C}$ chamber compared with the $+2$ and -10°C torpid groups (Fig. 4A). Thus, analyses of individual genes and of principal components indicates

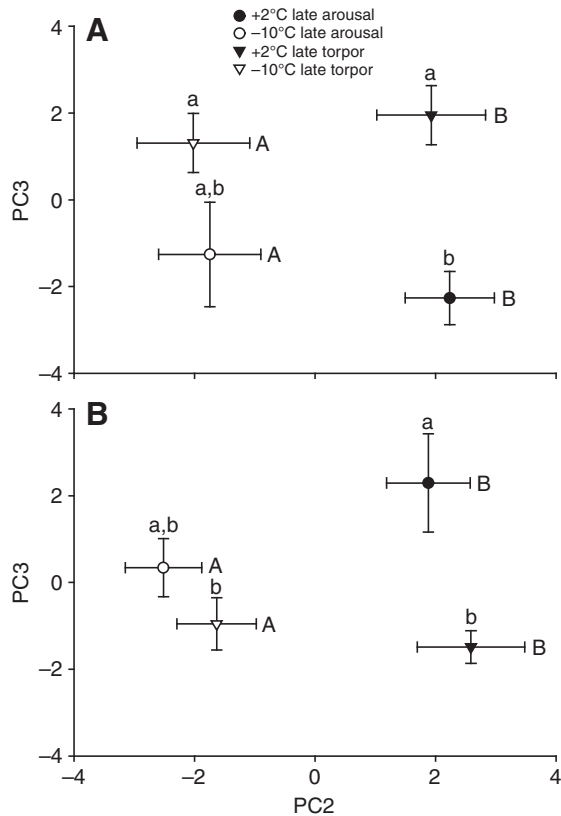


Fig. 4. Second and third principal components from a principal components analysis (PCA) on fold change in gene expression for (A) liver tissue and (B) brown adipose tissue from arctic ground squirrels sampled during torpor bouts and natural arousals within environmental chambers at +2 and -10°C . Uppercase letters indicate significant differences in PC2 whereas lowercase letters indicate differences in PC3. No significant group differences were found in the first principal component for either tissue.

a significant stage (torpid vs aroused) effect at $+2^{\circ}\text{C}$ (but not at -10°C) and a significant temperature effect in both torpid and aroused squirrels. However, our analyses suggest stage of hibernation and ambient temperatures explain only a small amount of the total variation in gene expression patterns observed among individuals.

In the second experiment, we also measured metabolic gene expression in BAT under the two different temperature regimes and compared hibernation states. Gene transcript levels in BAT were not significantly different among groups for any of the 50 genes tested (FDR-adjusted $P > 0.05$, supplementary material Table S4). The first, second and third PCs from a PCA on normalized gene expression levels accounted for 35.1, 14.3 and 9.0% of the total variation, respectively. We found no significant effect of group on PC1 ($F_{3,17}=0.36$, $P=0.78$), whereas group effects were significant for both PC2 ($F_{3,17}=12.03$, $P=0.0006$, $R^2=0.68$) and PC3 ($F_{3,17}=5.09$, $P=0.016$, $R^2=0.58$). *Post hoc* Tukey's tests indicated that PC2 values from torpid and aroused ground squirrels hibernating at -10°C were higher compared with both groups hibernating at $+2^{\circ}\text{C}$, whereas PC3 values were higher in aroused squirrels at $+2^{\circ}\text{C}$ compared with torpid squirrels in either temperature group (Fig. 4B). Thus, although we were unable to identify differentially expressed metabolic genes in BAT, our PCA results suggest that temperature and stage ($+2^{\circ}\text{C}$ animals only) had a small effect (explaining $<10\%$ of variance) on overall patterns of metabolic gene expression.

DISCUSSION

Perhaps uniquely among small hibernators, free-living arctic ground squirrels routinely defend large thermal gradients ($10\text{--}25^{\circ}\text{C}$) between T_b and T_a when torpid by substantially increasing metabolic rate while maintaining a subzero body temperature. Based on data from Buck and Barnes (Buck and Barnes, 2000), squirrels at -10°C would have had a steady-state torpid metabolic rate that is approximately ninefold higher than squirrels at $+2^{\circ}\text{C}$. Although we found significant effects of T_a and stage of hibernation (torpid vs after arousal) on metabolic gene expression in liver and BAT (based on PCA), the size of these effects was small and explained little of the observed variation in transcript levels. We identified a single gene (*OTC*) that differed among temperature groups; it was significantly lower at a T_a of -10°C compared with $+2^{\circ}\text{C}$. However, this difference was not confirmed in the second experiment, where *OTC* levels were slightly, albeit not significantly, higher in squirrels at -10°C compared with squirrels at $+2^{\circ}\text{C}$. In contrast, expression of a large number of metabolic genes in liver was substantially different during hibernation compared with the summer active period. In these comparisons, our results suggest that changes in expression of metabolic genes are primarily programmed by seasonal or circannual control, and are largely unaffected by increased thermogenesis and metabolic load associated with colder conditions. If low T_a elicits a switch from primarily lipid metabolism to mixed-fuel use, as was suggested in a previous study in arctic ground squirrels (Buck and Barnes, 2000), it does so without substantially altering the expression of these metabolic genes as detectable with this study design.

One possible explanation for the relatively small effect of T_a on patterns of gene expression is that arctic ground squirrels must be physiologically primed prior to entering torpor for decreases in T_a , including to below freezing, that occur during the torpor bout. During torpor, global suppression of mRNA transcription occurs (Van Breukelen and Martin, 2002; Morin and Storey, 2006). Furthermore, reversible phosphorylation of ribosomal initiation and elongation factors along with polysome disassociation results in global suppression of mRNA translation during torpor in brain, liver and kidney (Frerichs et al., 1998; Knight et al., 2000; Van Breukelen and Martin, 2001; Hittel and Storey, 2002). Thus, arctic ground squirrels would be unable to transcribe mRNA and translate proteins to respond to an increase in metabolic load associated with decreases in T_a that occurred within torpor bouts, which can last 24 days. Although soil temperatures (which we assume to be representative of T_a within the hibernacula) are much more stable than ambient conditions above the surface of the snow, decreases in soil temperature of up to 10°C can occur within the 18 day time frame of an average torpor bout (Buck and Barnes, 1999b).

Hittel and Storey found that BAT might be an exception to the general observation of arrested translation, as polyribosomes appear to remain intact and protein synthesis may continue during torpor (Hittel and Storey, 2002). Assuming protein translation is not suppressed to the same extent in BAT, it is surprising that hibernation state and T_a would have such a small effect on patterns of metabolic gene expression in this tissue if low T_a were causing a switch to use of non-lipid fuels. Thus, at the gene expression level, our molecular results appear inconsistent with the previous finding from respirometry that metabolism is increasingly fueled through non-lipid sources when ambient conditions drop below freezing (Buck and Barnes, 2000). However, it is also possible that gene expression at the mRNA level is not reflective of protein expression because of inhibitory controls of transcription, translation and protein

degradation during hypometabolism (Storey and Storey, 2004). For example, specific mRNA transcripts can be targeted by RNA-binding proteins, which influence their stability and translation at the post-transcriptional level. In addition, transcripts for metabolic genes become rapidly depleted following arousal (Yan et al., 2008). Direct comparisons of mRNA and protein levels for the same gene correlate at best with r -values of 0.6 in hibernating arctic ground squirrels and genes and proteins show no correlation at all when comparing squirrels in late torpor and those in early arousal ($r=0.065$) (Shao et al., 2010).

It is important to note that, although there is an approximately ninefold increase in metabolic rate between a T_a of 2 and -10°C , torpid metabolic rate at -10°C is only $\sim 20\%$ of the basal metabolic rate (Buck and Barnes, 2000). Thus, substantial modification of gene expression, and even protein expression, may not be necessary to support this level of metabolism. During natural arousal episodes, metabolic rate is only 46% higher in squirrels held at -12°C compared with individuals at $+2^\circ\text{C}$, and RQ values of ~ 0.78 for both of these treatment groups suggest mixed fuel metabolism (Karpovich et al., 2009). Switches in fuel use during torpor might be related to protein activity rather than changes in protein expression. Future studies measuring protein activity or substrate flux under varying ambient conditions would be useful in examining this possibility, although carrying this work out *in vivo* is not a simple task.

The increased metabolic rate associated with defending a thermal gradient could result in increased catabolism of protein stores independent of a fuel switch. Although hibernating ground squirrels are clearly adapted to preferentially catabolize lipid stores (reviewed in Carey et al., 2003), the muscles of 13-lined and golden-mantled ground squirrels atrophy slightly during hibernation, perhaps because of the long intervals of immobilization (Steffen et al., 1991; Wickler et al., 1991; Nowell et al., 2010). Thus, it is possible that the higher levels of protein loss observed in arctic ground squirrels are merely a function of their higher metabolic rates during torpor and/or interbout euthermia combined with a longer hibernation season. Such a scenario would suggest that instantaneous RQ values are not always indicative of fuel use, or that fuel use in our experiments differed from that in the previous study of Buck and Barnes (Buck and Barnes, 2000). RQ levels can be depressed as CO_2 accumulates in the blood during torpor re-entry (e.g. Malan et al., 1985; Nestler, 1990), but we are unaware of a mechanism for shifts in P_{CO_2} that could cause prolonged increases in RQ throughout steady-state torpor maintained over several weeks.

Seasonal changes

We found that the expression of metabolic genes in hibernators differs considerably from summer active animals, which is consistent with previous studies demonstrating extensive seasonal reorganization of the transcriptome in ground squirrels (Williams et al., 2005; Yan et al., 2008). Our results indicate that the reduction in carbohydrate metabolism and switch to lipid fuels during hibernation is achieved through an approximately sixfold increase in the expression of *CPT1A*, the rate-limiting enzyme in the β -oxidation of fatty acids (Drynan et al., 1996), a massive reduction in the expression of genes associated with fatty acid synthesis (*ACACB*, *SCD* and *ELOVL6*), and a more than fourfold increase in *FABP1*. With the exception of *CPT1A*, which had not previously been identified as an important mediator of the switch to lipid metabolism, differences in mRNA levels of these genes are consistent with previous genomic (Williams et al., 2005; Yan et al., 2008) and proteomic studies (Epperson et al., 2010; Shao et

al., 2010). The *FABP1* enzyme in hibernators is adapted to function in the intracellular binding and transport of fatty acids at low temperatures (Storey and Storey, 2004), indicating its importance in lipid metabolism during torpor. We also found that mRNA levels for the ketone-producing enzyme *HMGCS2* were significantly higher during hibernation compared with the summer active period, consistent with increased expression previously observed at the protein level in arctic (Shao et al., 2010), golden-mantled (Epperson et al., 2004) and 13-lined ground squirrels (Epperson et al., 2010).

The opposing effects of season on expression of the two *ACAT* isoforms observed in our study may seem counterintuitive. However, differences in expression are consistent with their different roles in lipid metabolism: *ACAT2* occurs in hepatocytes of the liver and its function relates to the secretion of very low density lipoprotein (VLDL) particles containing cholesteryl esters in their core, whereas *ACAT1* serves a more general role in cellular cholesterol homeostasis (Lee et al., 2000). Yan et al. also reported elevated levels of *ACAT2* during summer (Yan et al., 2008), which is likely associated with the secretion of VLDL particles from the liver as endogenous fatty acids are transported to peripheral tissues.

Phosphoenolpyruvate carboxykinase 1 (*PCK1*), a crucial enzyme in gluconeogenesis, was upregulated during hibernation. Gluconeogenesis occurs in the liver during arousal intervals to replenish glucose and glycogen stores depleted during torpor (Galster and Morrison, 1975; Serkova et al., 2007). The primary carbon skeletons used for gluconeogenesis are derived from pyruvate, lactate, glycerol and amino acids. Fatty acids are not converted to glucose, as the two carbon unit of acetyl-CoA derived from β -oxidation is lost as CO_2 following its incorporation into the TCA cycle. Thus, glycerol is the only substrate derived from the breakdown of triglycerides that can be utilized for gluconeogenesis. Increased expression of *PCK1*, which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, would only increase gluconeogenesis from pyruvate, lactate or amino acids because glycerol enters the gluconeogenic pathway downstream of *PCK1*.

Stage effects

Consistent with previous studies of gene expression during hibernation (e.g. Williams et al., 2005), we found only a small effect of the torpor–arousal cycle on gene expression in liver and BAT of hibernating arctic ground squirrels. We found that mRNA levels for *GSY1* and *GSK3A* were upregulated during arousals in liver tissue at 2°C ; Yan et al. also reported elevated *GSK3A* during arousal (Yan et al., 2008). Glycogen in liver and thigh muscle was found to decline steadily over the course of a torpor bout, but remain constant in kidney, brown fat and heart (Galster and Morrison, 1970). Thus, elevated *GSY1* and *GSK3A* levels may reflect the need to replenish glycogen stores from the glucose generated during arousals.

Conclusions

We found that mRNA levels of metabolic genes were consistent with a switch from a carbohydrate-based metabolism during the summer active season to lipid fuels during hibernation. Ambient temperature had only a small effect on patterns of gene expression, a finding that does not support a hypothesized switch to non-lipid fuels when ambient conditions drop below freezing. However, mRNA levels explained only 40% of the variation in protein expression levels (Shao et al., 2010) and it is possible that translational controls or post-translational modifications (which alter protein activity) are responsible for switches in fuel use patterns that have previously been inferred based on shifts in RQ.

LIST OF ABBREVIATIONS

BAT	brown adipose tissue
FDR	false discovery rate
PCA	principal component analysis
qRT-PCR	quantitative reverse-transcription PCR
RQ	respiratory quotient
T_a	ambient temperature
T_b	body temperature
TCA cycle	tricarboxylic acid cycle
VLDL	very low density lipoprotein

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REFERENCES

- Andersen, C. L., Jensen, J. L. and Orntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245-5250.
- Barnes, B. M. (1989). Freeze avoidance in a mammal: body temperatures below 0°C in an arctic hibernator. *Science* **244**, 1593-1595.
- Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289-300.
- Buck, C. L. and Barnes, B. M. (1999a). Annual cycle of body composition and hibernation in free-living arctic ground squirrels. *J. Mammal.* **80**, 430-442.
- Buck, C. L. and Barnes, B. M. (1999b). Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter. *J. Mammal.* **80**, 1264-1276.
- Buck, C. L. and Barnes, B. M. (2000). Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279**, R255-R262.
- Carey, H. V., Andrews, M. T. and Martin, S. L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83**, 1153-1181.
- Drynan, L., Auant, P. A. and Zammit, V. A. (1996). Flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over β -oxidation, ketogenesis and tricarboxylic acid cycle activity in hepatocytes isolated from rats in different metabolic states. *Biochem. J.* **323**, 119-122.
- Epperson, L. E., Dahl, T. A. and Martin, S. L. (2004). Quantitative analysis of liver protein expression during hibernation in the golden-mantled ground squirrel. *Mol. Cell. Proteomics* **3**, 920-933.
- Epperson, L. E., Rose, J. C., Carey, H. V. and Martin, S. L. (2010). Seasonal proteomic changes reveal molecular adaptations to preserve and replenish liver proteins during ground squirrel hibernation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R329-R340.
- Ferron, J. (1996). How do woodchucks (*Marmota monax*) cope with harsh winter conditions? *J. Mammal.* **77**, 412-416.
- Fleige, S. and Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Mol. Aspects Med.* **27**, 126-139.
- Frerichs, K. U., Smith, C. B., Brenner, M., DeGracia, D. J., Krause, G. S., Marrone, L., Dever, T. E. and Hallenbeck, J. M. (1998). Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci. USA* **95**, 14511-14516.
- Galster, W. and Morrison, P. R. (1970). Cyclic changes in carbohydrate concentrations during hibernation in the arctic ground squirrel. *Am. J. Physiol.* **218**, 1228-1232.
- Galster, W. and Morrison, P. R. (1975). Gluconeogenesis in arctic ground squirrels between periods of hibernation. *Am. J. Physiol.* **228**, 325-330.
- Geiser, F. (2004). Metabolic rate and body temperature reduction during hibernation and daily torpor. *Annu. Rev. Physiol.* **66**, 239-274.
- Geiser, F. and Ruf, T. (1995). Hibernation versus daily torpor in mammals and birds: physiological variables and classification of torpor patterns. *Physiol. Zool.* **68**, 935-966.
- Gorer, P. A. (1930). The physiology of hibernation. *Biol. Rev.* **5**, 213-230.
- Hittel, D. and Storey, K. B. (2002). The translation state of differentially expressed mRNAs in the hibernating 13-lined ground squirrel (*Spermophilus tridecemlineatus*). *Arch. Biochem. Biophys.* **401**, 244-254.
- Karpovich, S. A., Toien, O., Buck, C. L. and Barnes, B. M. (2009). Energetics of arousal episodes in hibernating arctic ground squirrels. *J. Comp. Physiol. B* **179**, 691-700.
- Kenagy, G. J., Sharbaugh, S. M. and Nagy, K. A. (1989). Annual cycle of energy and time expenditure in a golden mantled ground squirrel population. *Oecologia* **78**, 269-282.
- Knight, J. E., Narus, E. N., Martin, S. L., Jacobson, A., Barnes, B. M. and Boyer, B. B. (2000). mRNA stability and polysome loss in hibernating arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* **20**, 6374-6379.
- Lee, R. G., Willingham, M. C., Davis, M. A., Skinner, K. A. and Rude, R. L. (2000). Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J. Lipid Res.* **41**, 1991-2001.
- Lee, T. N., Barnes, B. M. and Buck, C. L. (2009). Body temperature patterns during hibernation in a free-living Alaska marmot (*Marmota flaviventris*). *Ethol. Ecol. Evol.* **21**, 403-413.
- Long, R. A., Hut, R. A. and Barnes, B. M. (2007). Simultaneous collection of body temperature and activity data in burrowing mammals: a new technique. *J. Wildl. Manage.* **71**, 1375-1379.
- Lyman, P. C. and Chatfield, P. O. (1955). Physiology of hibernation in mammals. *Physiol. Rev.* **35**, 403-425.
- Malan, A., Rodeau, J. L. and Daull, F. (1985). Intracellular pH in hibernation and respiratory acidosis in the European hamster. *J. Comp. Physiol. B* **156**, 251-258.
- Michener, G. R. (1992). Sexual differences in over-winter torpor patterns of Richardson's ground squirrels in natural hibernacula. *Oecologia* **89**, 397-406.
- Morin, P., Jr and Storey, K. B. (2006). Evidence for a reduced transcriptional state during hibernation in ground squirrels. *Cryobiology* **53**, 310-318.
- Nestler, J. R. (1990). Relationships between respiratory quotient and metabolic rate during entry to and arousal from daily torpor in deer mice (*Peromyscus maniculatus*). *Physiol. Zool.* **63**, 504-514.
- Nowell, M. M., Choi, H. and Rourke, B. C. (2010). Muscle plasticity in hibernating ground squirrels (*Spermophilus lateralis*) is induced by seasonal, but not low-temperature, mechanisms. *J. Comp. Physiol. B* **181**, 147-164.
- Otis, J. P., Ackermann, L. W., Denning, G. M. and Carey, H. V. (2010). Identification of qRT-PCR reference genes for analysis of opioid gene expression in a hibernator. *J. Comp. Physiol. B* **180**, 619-629.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
- Ringnér, M. (2008). What is principal component analysis? *Nat. Biotechnol.* **26**, 303-304.
- Serkova, N. J., Rose, J. C., Epperson, L. E., Carey, H. V. and Martin, S. L. (2007). Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. *Physiol. Genomics* **31**, 15-24.
- Shao, C., Liu, Y., Ruan, H., Li, Y., Wang, H., Kohl, F., Goropashnaya, A. V., Fedorov, V. B., Zeng, R., Barnes, B. M. and Yan, J. (2010). Shotgun proteomics analysis of hibernating arctic ground squirrels. *Mol. Cell. Proteomics* **9**, 313-326.
- Steffen, J. M., Koebel, D. A., Musacchia, X. J. and Milsom, W. K. (1991). Morphometric and metabolic indices of disuse in muscles of hibernating ground squirrels. *Comp. Biochem. Physiol.* **99B**, 815-819.
- Storey, K. B. and Storey, J. M. (2004). Metabolic rate depression in animals: transcriptional and translational controls. *Biol. Rev.* **79**, 207-233.
- Van Breukelen, F. and Martin, S. (2001). Translational initiation is uncoupled from elongation at 18°C during mammalian hibernation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281**, R1374-R1379.
- Van Breukelen, F. and Martin, S. (2002). Reversible depression of transcription during hibernation. *J. Comp. Physiol. B* **172**, 355-361.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, research0034.1-0034.11.
- Wickler, S. J., Hoyt, D. F. and Van Breukelen, F. (1991). Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **261**, 1214-1217.
- Williams, D. R., Epperson, L. E., Li, W., Hughes, M. A., Taylor, R., Rogers, J., Martin, S. L., Cossins, A. R. and Gracey, A. Y. (2005). Seasonally hibernating phenotype assessed through transcript screening. *Physiol. Genomics* **24**, 13-22.
- Yan, J., Burman, A., Nichols, C., Alila, L., Showe, L. C., Showe, M. K., Boyer, B. B., Barnes, B. M. and Marr, T. G. (2006). Detection of differential gene expression in brown adipose tissue of hibernating arctic ground squirrels with mouse microarrays. *Physiol. Genomics* **25**, 346-353.
- Yan, J., Barnes, B. M., Kohl, F. and Marr, T. G. (2008). Modulation of gene expression in hibernating arctic ground squirrels. *Physiol. Genomics* **32**, 170-181.
- Young, P. J. (1990). Hibernating patterns of free-ranging Columbian ground squirrels. *Oecologia* **83**, 504-511.