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RESEARCH ARTICLE

Liver proteome response to torpor in a basoendothermic mammal, *Tenrec ecaudatus*, provides insights into the evolution of homeothermy

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Abstract

Many mammals use adaptive heterothermy (e.g., torpor, hibernation) to reduce metabolic demands of maintaining high body temperature (T_b). Torpor is typically characterized by coordinated declines in T_b and metabolic rate (MR) followed by active rewarming. Most hibernators experience periods of euthermy between bouts of torpor during which homeostatic processes are restored. In contrast, the common tenrec, a basoendothermic Afrotherian mammal, hibernates without interbout arousals and displays extreme flexibility in T_b and MR. We investigated the molecular basis of this plasticity in tenrecs by profiling the liver proteome of animals that were active or torpid with high and more stable T_b (~32°C) or lower T_b (~14°C). We identified 768 tenrec liver proteins, of which 50.9% were differentially abundant between torpid and active animals. Protein abundance was significantly more variable in active cold and torpid compared with active warm animals, suggesting poor control of proteostasis. Our data suggest that torpor in tenrecs may lead to mismatches in protein pools due to poor coordination of anabolic and catabolic processes. We propose that the evolution of endothermy leading to a more realized homeothermy of boreoeutherians likely led to greater coordination of homeostatic processes and reduced mismatches in thermal sensitivities of metabolic pathways.

hibernation; liver; proteome; tenrec; torpor

INTRODUCTION

Mammalian heterothermy likely represents the plesiomorphic (ancestral) state with mammals displaying a core body temperature (T_b) below 35°C classified as being protoendothermic or basoendothermic (1). The increased endothermy leading to a more realized homeothermic state is considered to be the apomorphic (derived) state and is prevalent in the boreoeutherian clade of placental mammals, for example, "modern" mammals wherein active animal core T_b is relatively high and stable. Why increased homeothermy evolved in mammals has puzzled researchers for decades and has prompted numerous compelling models for the evolution of endothermy. These models suggest that homeothermy may have enabled mammals to occupy new niches (niche expansion hypothesis), increase locomotor activities (aerobic capacity model), reduce body size (body size reduction hypothesis), enhance digestion efficiency (assimilation capacity model), or increase resource allocation to offspring (parental care model) (2). All of these models are cogent but experimentally supporting why increased endothermy evolved is difficult (3). Most attempts to address the veracity of the models have used systems such as mice or rats, in which T_b and metabolism are linked and very constrained and fluctuation of core T_b is minimal.

Many extant mammals are heterotherms, which use torpor or hibernation to reduce the metabolic costs of maintaining high T_b during periods of environmental stress (e.g., resource limitation, cold temperature; 4). It has been proposed that the evolution of homeothermy occurred via heterothermy and that the latter enabled small mammals to survive the Cretaceous-Paleogene extinction event, with extant heterotherms present in monotreme, marsupial, and placental mammal lineages (2). Periods of heterothermy in mammals may range from daily torpor bouts to months of deep seasonal hibernation. Torpor is typically defined as a highly controlled and coordinated decline in physical activity, heart, respiration, and metabolic rates and T_b, which may approach ambient temperature (T_a) (4). Although the extent of T_b depression varies between species, many common metabolic adjustments to torpor have been identified, including reductions in protein and nucleic acid synthesis and a switch from carbohydrate to lipid and ketone metabolism (5). Deep seasonal hibernators such as ground squirrels maintain homeostasis during prolonged hibernation by undergoing regular periods of rewarming to normothermic T_b (interbout arousals, IBA) between torpor bouts that allow for homeostatic processes like protein synthesis and degradation and enable animals to remove metabolic wastes, replenish energy

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substrates, and recover immune function, among other benefits (4).

We recently described remarkable thermoregulatory and metabolic flexibility in the common tenrec, *Tenrec ecaudatus* (6). Tenrecs are Afrotherian placental mammals thought to have radiated from a common ancestor that landed on Madagascar some 30 to 56 Ma (7). Tenrecs have numerous morphological characteristics seemingly more ancestral than even what *Schrewdinger*, the hypothetical, reconstructed, placental mammal ancestor, was posited to possess (8). These features, which include the presence of a very small and lissencephalic brain, cloaca, and internal testes and a lack of zygomatic arches and tympanic bullae, suggest that tenrecs may serve as an extant representative of an early placental mammalian ancestor (6, 9). Wild tenrecs have been shown to hibernate in burrows for up to 9 mo, during which they experience continuous torpor with no IBA characterized by T_b that tracks soil temperature (10). Our laboratory experiments found that common tenrecs housed at 12°C to 28°C could be active or torpid at any of these temperatures. What was remarkable, however, was that both active and torpid T_b may approach within 1°C of T_a. Active tenrecs have variable T_b such that animals that are housed at 12°C may have a core T_b of ~13°C to 28°C yet still be fully ambulatory (active season tenrecs with a core T_b of ~13°C may even swim). Accompanying this remarkable thermal plasticity were variable oxygen consumption rates; active season tenrecs housed at 12°C have a 25-fold variation in resting oxygen consumption rate with low metabolic rates being indistinguishable from that of a torpid tenrec. We demonstrated that such variation is even semi-independent of T_b or heart rate (6). During the hibernation season, tenrecs are consistently torpid but the extent of lethargy during torpor is somewhat dependent on the time of year and T_a. Animals sampled in June and/or at 12°C are usually more lethargic and ataxic than animals sampled early in the hibernation season or at 28°C. During the hibernation season, T_b approximates T_a to within 1°C and does not fluctuate significantly over time (weeks or months), and tenrecs are extremely lethargic and difficult to arouse (6). If tenrecs are indeed representative of an ancestral placental mammal and experience pronounced thermal plasticity, perhaps understanding the implication of that plasticity may lead to gained insight into the evolution of endothermy.

We focused our first investigation of the molecular underpinnings of thermoregulatory and metabolic plasticity in tenrecs by profiling the proteome of the liver, the nexus of metabolic coordination in animals. The liver is a protein-rich and metabolically active tissue that has been extensively studied at the proteome level in other hibernating species, providing insights into metabolic shifts and coordination of homeostatic processes (e.g., protein turnover) during torpor (11). We compared the liver proteomes of tenrecs that were either active or hibernating (torpid) with a high and more stable T_b or a lower T_b as an indicator of how well the proteome was being regulated. We describe protein abundance differences between active and torpid tenrecs that are conserved with those observed in other hibernators and suggest common metabolic shifts during torpor. However, we also found high levels of variability in protein abundance in cold active and torpid tenrecs, which was dampened in active animals that maintained a higher T_b. We propose that the evolution of endothermy leading to a more realized homeothermy, with an associated increase in oxygen consumption rates, allowed for increased coordination of cellular processes, leading to greater homeostasis in mammals.

MATERIALS AND METHODS

Study Subjects and Husbandry

All experimental procedures were approved by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee. Our colony of common tenrecs, *Tenrec ecaudatus*, are derived from 40 wild-caught tenrecs imported under federal and state permits from Mauritius in June 2014. The animals used in this study were of mixed sex (6 males, 9 females) and mass (570 ± 52 g; means ± SE). Tenrecs were maintained on Mazuri insectivore diet (Purina) supplemented with dry dog food or wet cat food and were weighed regularly to monitor health. All tenrecs were maintained on 12:12 light/dark cycle; for sampling, animals were individually housed in rat cages within an environmental chamber for temperature control. Body temperature was monitored using precalibrated iButton temperature loggers (DS1922L; Maxim) held against the chest by specially designed harnesses to ensure animals were torpid or active. Torpid animals were sampled in June when animals are the most lethargic and resistant to disturbance (6). Torpid animals were lethargic and T_b approximated T_a to within 1°C. These animals were observed to be in torpor (prolonged lethargy and anorexia) for several weeks before sampling (6). T_b was confirmed by placing a thermocouple against the liver during tissue collection. Active tenrecs were euthanized by CO_2 asphyxiation followed by cervical dislocation. Torpid tenrecs were euthanized by cervical dislocation as their low respiratory and heart rates precluded use of anesthetic or CO_2. Animals were dissected on ice, and tissues were snap-frozen in liquid N_2. Samples were stored at −80°C until processing. We analyzed five sample groups with three animals (n = 3) per group (Fig. 1): active T_a warm-T_b warm (T_a = 28°C; $T_{b\text{-liver}}$ =

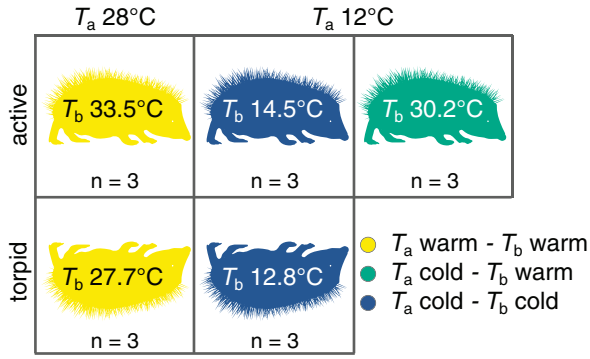

Figure 1. Diagram illustrating the experimental design used in the study. Common tenrecs (*Tenrec ecaudatus*) were housed at ambient temperature (T_a) of either 12°C (T_a cold) or 28°C (T_a warm) and had mean body temperatures (T_b) shown in the diagram at time of sampling. Three groups were active, whereas two groups were torpid at the time of sampling (n = 3 animals per group). Animal image was obtained and modified from Pixabay under the Pixabay License.

33.5 ± 0.3°C), active T_a cold-T_b warm (T_a = 12°C; $T_{b\text{-liver}}$ = 30.2 ± 2.3°C), active T_a cold-T_b cold ($T_{b\text{-liver}}$ = 14.5 ± 1.0°C), torpid T_a warm-T_b warm ($T_{b\text{-liver}}$ = 27.7 ± 0.2°C), and torpid T_a cold-T_b cold ($T_{b\text{-liver}}$ = 12.8 ± 0.1°C; Fig. 1).

Sample Preparation

Liver proteins were extracted and digested with trypsin using previously reported protocols (12). Specifically, ~50 mg of each frozen liver sample were minced on dry ice and homogenized by bead beating in 1 mL of denaturing buffer [1% wt/vol sodium deoxycholate (SDC), 8 M urea, 5 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate] in a Bullet Blender Storm 24 (Next Advance; 2 cycles of 2 min at power 12). Homogenates were denatured for 1 h at 37°C and alkylated with 15 mM iodoacetamide in the dark at room temperature for 30 min. Alkylation was quenched with 5 mM DTT. Samples were diluted to reduce urea concentration to <1 M, and protein concentration was estimated using the Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins were digested in solution using Trypsin Gold (Mass Spectrometry Grade, Promega) at 1:125 μg enzyme-to-protein ratio for 16 h at 37°C. Digested peptides were acidified to pH < 2 to inactivate trypsin and precipitate SDC, desalted using Pierce C18 Spin Columns (Thermo Scientific), lyophilized, and resuspended in 0.1% formic acid in LC/MS-grade water. Peptide concentration was determined using the Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific). Samples were assayed in triplicate (coefficient of variation, CV < 3.5%). Sample concentrations were determined using a linear fit for the standard curve (R^2 = 0.993).

LC-MS/MS

Peptide samples were diluted to 200 ng/μL with 0.1% formic acid in LC/MS-grade water, and 5 μL were loop injected by a Dionex Ultimate 3000 autosampler onto a reversed-phase trap column (Acclaim PepMap 100 C18 LC column; 75 μm id × 2 cm, 3 μm particle size, 100 A pore size, Thermo Fisher Scientific). Peptides were eluted onto a reversed-phase analytical column (EASY-Spray C18 LC column; 75 μm id × 15 cm, 100 A, Thermo Fisher Scientific) held at 35°C for HPLC. Solvents A and B were 0.1% formic acid in water and in acetonitrile, respectively. Solvent B was used as following: 3% for 5 min, 3%–28% for 75 min, 28%–45% for 25 min, 45%–95% for 5 min, 95% for 5 min, return to 3% for 5 min, and followed by 2% for 25 min. Flow rates were held at 300 nL/min with each sequencing run set to 140 min. Mass spectrometry analysis was performed using Orbitrap Fusion Tribrid mass spectrometer equipped with an EASY-Spray ion source (Thermo Fisher Scientific) operated in a data-dependent acquisition (DDA) manner by Xcalibur 4.0 software (Thermo Fisher Scientific). Instrument and data acquisition settings were the same as those previously reported (12). The highest-intensity precursor ions in respective elution profiles were quadrupole filtered and fragmented using high collision dissociation (HCD). MS1 precursors and MS2 product ions were resolved using the orbitrap mass analyzer.

Data Analysis

Raw spectrum files were uploaded to the Proteome Xchange MassIVE database (doi:10.25345/C51J6M, https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=0aed8ad3207b4006bd86b41a229f499f). Protein identification using the UniProtKB Afrotheria database (Taxonomy ID: 311790; 61,199 entries, downloaded on June 25, 2020) and label-free quantification (LFQ) were performed using MaxQuant v1.6.14.0 with default settings. Technical replicates for each biological replicate ("experiment") were analyzed as part of the same experiment; the median abundance values for three technical replicates were reported. The "match between runs" function was enabled with a matching time window of 0.7 min and alignment time window of 20 min. Carbamidomethylation (C) was selected as a fixed modification and oxidation (M) and deamidation (NQ) were selected as variable modifications; a maximum of three modifications per peptide were allowed. False discovery rate (FDR) was determined by searching reversed Afrotheria and MaxQuant contaminant databases. Only hits below 1% FDR were retained for further analyses; hits to contaminant or reversed databases were removed. Of a total 1,162,457 MS/MS spectra generated in this experiment, 316,852 (27.3%) had matches to the Afrotheria protein database. Protein LFQ abundance values were $\log_2$-transformed after removing proteins with <2 unique peptides and ≥2 missing values per sample group. Remaining missing values were imputed using the llsImpute function (k = 150) in the pcaMethods Bioconductor package (13, 14) using R v3.6.0 and RStudio v1.1.453 (15, 16). Differential protein abundance analyses were conducted using limma v3.40.6 (17); R code is provided as a Supplemental Data (see https://doi.org/10.6084/m9.figshare.14745279.v1). Proteins with abundances that differed by more than 20% between groups were considered differentially abundant at Benjamini–Hochberg adjusted $P <$ 0.05. Protein abundance data were summarized using pheatmap R package (18) with complete clustering of both rows and columns by Euclidean distance. Principal components analysis (PCA) was conducted using the prcomp function in the stats package and visualized using factoextra (19). Functional enrichment analysis was conducted using Enrichr (20) and the human WikiPathways 2019 database (21). Differentially abundant proteins were mapped to the human WikiPathways database (7,601 proteins, updated January 28, 2021) using Cytoscape 3.8.0 (22). Functional enrichment networks were created using ClueGO v2.5.7 in Cytoscape (23). Right-sided hypergeometric test was used to identify significantly enriched pathways at adjusted $P < 0.05$. The κ score threshold was 0.5; redundant groups with >50.0% overlap were merged.

RESULTS

We used LC-MS/MS to profile the liver proteome of common tenrecs housed at ambient temperatures of 12°C (T_a cold) or 28°C (T_a warm) that had liver temperatures of 12.6°C to 15.5°C (T_b cold) or 27.5°C to 33.8°C (T_b warm) and were either active or torpid at the time of sampling (Fig. 1). We identified 7,181 peptides, 1,526 protein groups, and 999 proteins with ≥2 unique peptides (Supplemental File 1; see https://doi.org/10.6084/m9.figshare.14721315.v1). LFQ was used to obtain protein abundance values based on precursor ion intensity; 768 proteins with ≤1 missing value per group were used for differential protein abundance analyses (Supplemental File 2; see https://doi.org/10.6084/m9.figshare.14745255.v1; Fig. 3). There was notable individual variability

in protein abundance among torpid animals (at both temperatures) and among active cold animals, whereas the active warm group showed the least variability between replicates (Figs. 2 and 3). One active tenrec at T_a cold-T_b cold (TE01) grouped more closely with torpid animals, whereas one active tenrec at T_a cold-T_b warm (TE05) clustered with T_b cold animals (Fig. 2).

The proteome response to torpor was dramatic: 391 proteins (50.9%) were differentially abundant between all active and all torpid animals (Fig. 4 and Supplemental File 3; see https://doi.org/10.6084/m9.figshare.14745270.v1). Proteins that increased in abundance in active tenrecs (n = 179) belonged to 57 overrepresented biological pathways (adj. $P <$ 0.05; Fig. 5 and Supplemental File 4; see https://doi.org/10.6084/m9.figshare.14745273.v1), which included electron transport chain (ETC), amino acid metabolism, tryptophan metabolism, tricarboxylic acid (TCA) cycle, nuclear receptors metapathway, metapathway biotransformation phase I and II, and cytosolic ribosomal proteins, among others. The most highly increased proteins in active tenrecs included amino acid metabolism enzymes MAOB, ALDH4A1, and AASS; ornithine-citrulline antiporter SLC25A15; and fatty acid transporter ABCD3. Proteins that increased in torpid tenrecs (n = 212) belonged to 44 overrepresented pathways (Fig. 5 and Supplemental File 4), which included proteasome degradation, parkin-ubiquitin proteasomal system pathway, focal adhesion, VEGFA-VEGFR2 signaling pathway, calcium regulation, and glycolysis and gluconeogenesis, among others. The most highly increased proteins in torpid tenrecs included detoxification enzymes NQO1 and AOX3, calcium binding protein S100A10, scaffolding protein AHNAK, and acute-phase protein A2M.

One major advantage of the tenrec model is the ability to separate out the effects of temperature from metabolic state —warm torpid or cold active animals allow comparisons that are otherwise unavailable for any other hibernator. To determine whether proteome responses to hibernation varied with temperature, we compared proteomes of active and torpid tenrecs within the same temperature groups. In general, warm tenrecs demonstrated a more pronounced proteome response to hibernation compared with cold tenrecs: there were 310 differentially abundant proteins (DAPs) between active and torpid tenrecs at T_a warm-T_b warm and 120 DAPs between active and torpid tenrecs at T_a cold-T_b cold; 95 of these were common in both temperature groups (Supplemental File 3).

To determine whether variability in T_b or exposure to cold T_a influenced protein abundance, we compared protein abundance between tenrecs that differed only by T_b or T_a. Ten proteins had altered abundance between tenrecs housed at T_a cold and those at T_a warm (Fig. 6 and Supplemental File 3). Proteins that increased at T_a cold included acute-phase proteins (HP, MPO), RNA processing enzymes (DDX3Y, DDX5, HNRNPH2), fibrinogen chains (FGA, FGG), histone protein BZW1, and translation initiation factor EIF4A1. The only protein that increased at T_a warm was

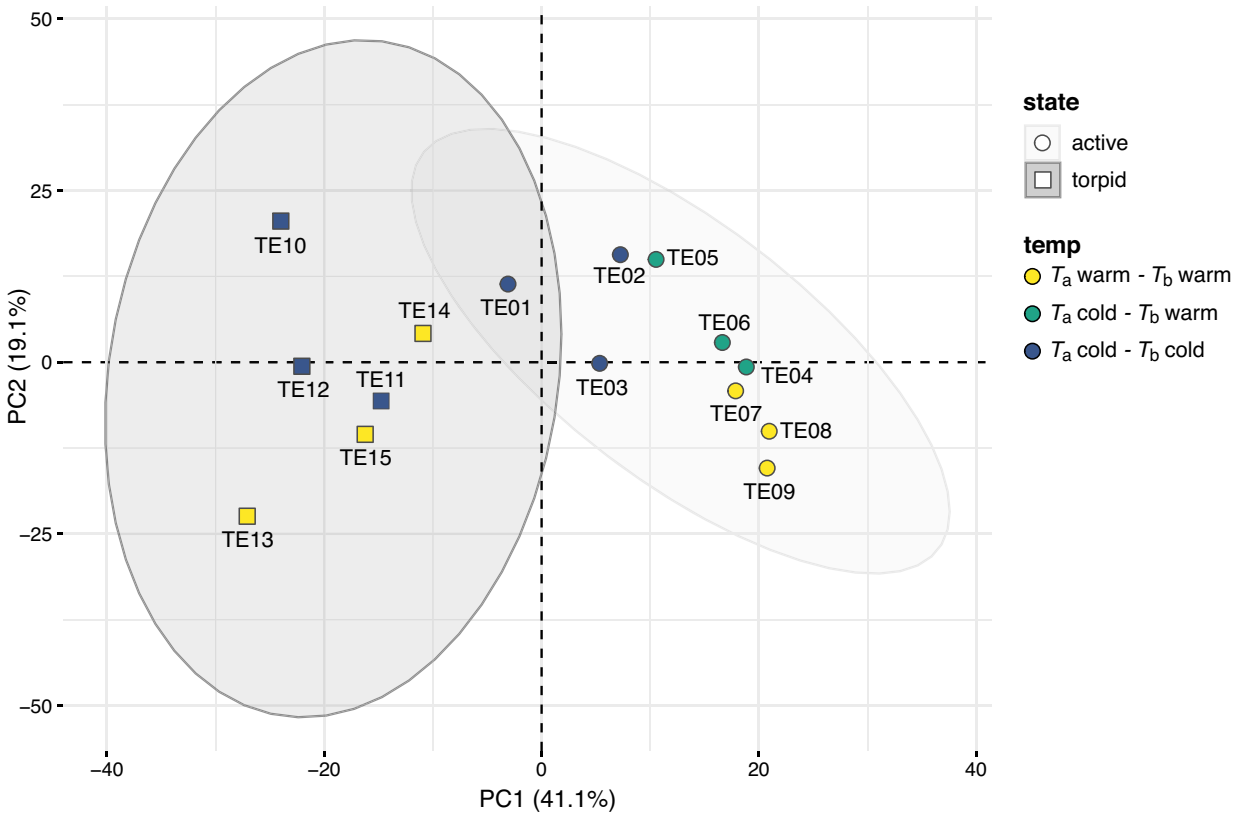

Figure 2. Principal components analysis (PCA) plot of liver proteome data from 5 groups of common tenrecs sampled at varying ambient and body temperatures (temp; T_a and T_b, respectively) and activity states (state; active and torpid). Ellipses show 95% confidence intervals for active and torpid groups. PC1 and PC2, principal component 1 and 2.

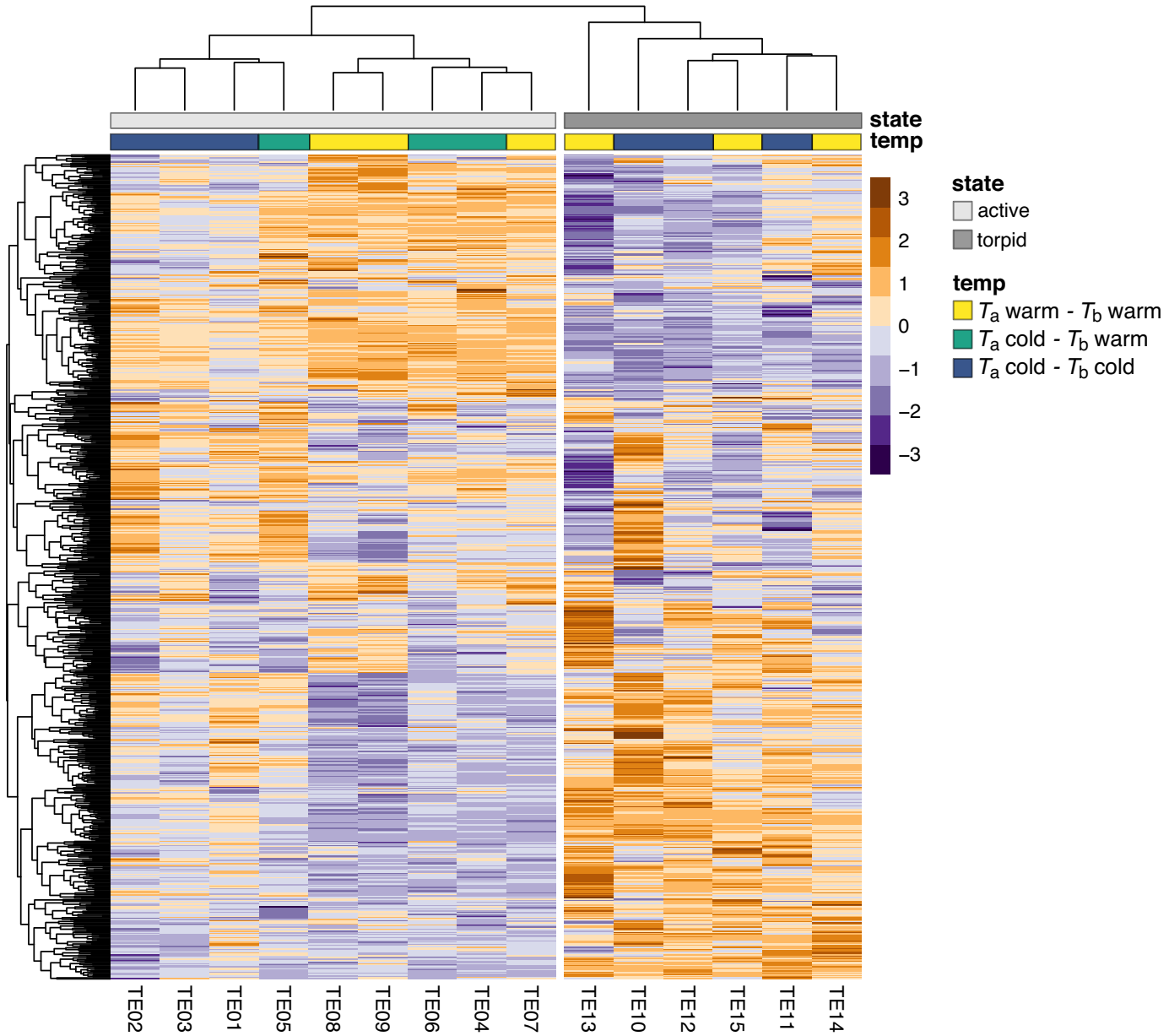

Figure 3. Heatmap showing scaled abundance of 768 proteins (rows) in liver of 5 groups of common tenrecs (columns) sampled at varying ambient and body temperatures (temp; T_a and T_b, respectively) and activity states (state; active and torpid). Rows and columns were clustered by Euclidean distance.

histidine ammonia-lyase (HAL). There were no DAPs between T_b cold and T_b warm tenrecs. We then examined the effect of temperature on protein abundance while controlling for activity state. There were 58 DAPs between active T_a warm-T_b warm and active T_a cold-T_b cold animals (Supplemental File 3). Proteins with higher abundance in the warm active group (n = 22) were associated with metapathway biotransformation, fatty acid oxidation, urea cycle, amino acid metabolism, steroid metabolism, and redox homeostasis, among other functions. Proteins that increased in abundance in the cold active group (n = 36 proteins) included translation initiation factors, cold-inducible RNA-binding proteins, and proteins associated with glycolysis, fatty acid synthesis, and calcium binding. Notably, 26 DAPs were also increased during torpor in warm tenrecs and 6 during torpor in cold tenrecs (Table 1). Haptoglobin was the only protein that increased in active tenrecs at T_a cold-T_b warm relative to active tenrecs at T_a warm-T_b warm. There were no DAPs between cold and warm torpid tenrecs.

DISCUSSION

Metabolic reactions are very sensitive to changes in temperature. For every 10°C change, one expects to see a two- to threefold change in enzymatic activity or oxygen consumption despite the only ~4% change in thermal energy (Q_{10} effects; 24). Moreover, many enzymes are known to be particularly thermally sensitive or insensitive. Since thermal sensitivities of enzymes vary even within a single metabolic pathway, changes in T_b would make coordination of

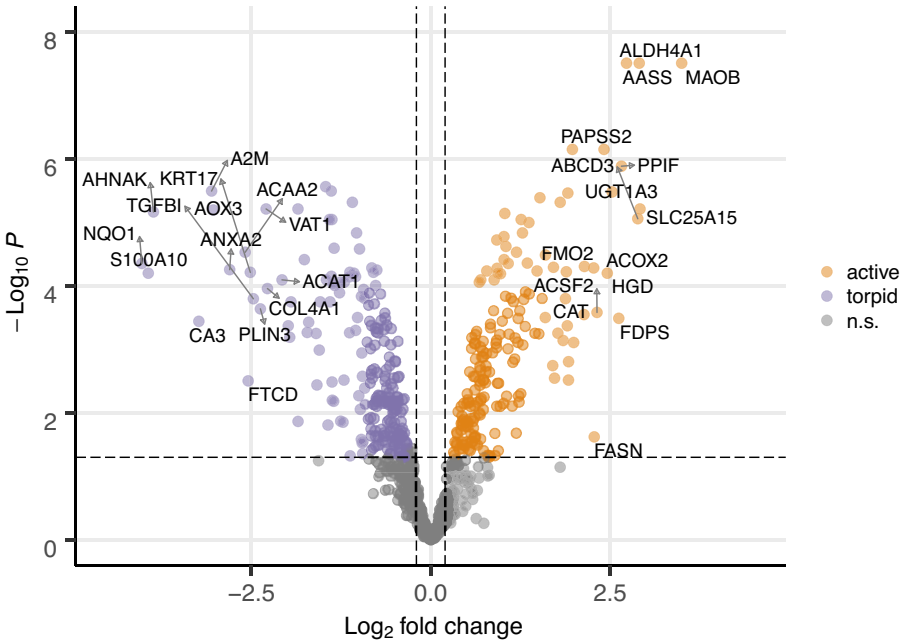

Figure 4. Volcano plot showing proteins that were differentially abundant between active ($n = 9$ animals) and torpid ($n = 6$ animals) common tenrecs. Proteins that were significantly increased (fold change > 1.2, adjusted $P < 0.05$) in active relative to torpid animals are shown in orange, whereas those that were significantly increased in torpid animals are shown in purple. Proteins shown in gray were not considered differentially abundant. Gene symbols for the top 15 most highly increased proteins in each group are shown.

homeostatic processes difficult. Although the thermal plasticity of common tenrecs is extraordinary, even in other monotremes, marsupials, Xenarthrans, and Afrotherians, T_b can vary by as much as 8°C and T_b is often much lower than ~37°C (25–29). In other words, the high and relatively stable T_b of boreoeutherian mammals might be considered an apomorphic trait (1). We contend this apomorphy allowed for more coordination of homeostatic pathways.

The implications of exploiting pronounced heterothermy are tremendous. For instance, most small hibernators like ground squirrels avail themselves of low temperatures to effectively use torpor. Torpid ground squirrel T_b approaches that of ambient temperature and may even be below 0°C (4). Perhaps it is not suprising that oxygen consumption rates in torpid ground squirrels may be as low as 1/100th of active rates. We have argued that this profound metabolic depression was afforded by placing energetically costly but seemingly vital processes for homeostasis such as transcription, translation, and protein degradation on hold during torpor (30). Virtually all hibernators experience periods of euthermy between bouts of torpor during which those homeostatic processes are restored (4). In other words, the hibernation season of most mammals is characterized by periods of torpor during which most homeostatic processes (e.g., macromolecule synthesis) are slowed, interrupted by periods of euthermy wherein homeostasis is restored.

In contrast, common tenrecs exhibit remarkable plasticity of thermoregulation and metabolism (6). Tenrecs may be fully active or torpid at either high or low ambient/body temperatures. Furthermore, T_b is a poor predictor of oxygen consumption rate because animals may have the same T_b with widely varying oxygen consumption rates. However, the variation seen in metabolism is exacerbated by temperature. Although animals housed at 12°C experience as much as a ~25-fold variation in resting oxygen consumption rate, animals housed at 20°C experience only an approximately fivefold variation. The variation is further reduced at 28°C. For instance, at T_a 12°C, oxygen consumption rates in active tenrecs were as low as 0.0405 ± 0.021 (means ± SE) mL $O_2 \cdot g^{-1} \cdot h^{-1}$ (active low; T_b = 18.1 ± 7.0°C, means ± SD) or as high as 0.9916 ± 0.052 mL $O_2 \cdot g^{-1} \cdot h^{-1}$ (active high; T_b = 28.0 ± 3.4°C, n = 8) for a 60-min period of stable oxygen consumption (6). For comparison, torpid tenrec oxygen consumption rates at 12°C were 0.0115 ± 0.023 mL $O_2 \cdot g^{-1} \cdot h^{-1}$ and rival those of torpid Arctic ground squirrels housed at 4°C (0.012 mL $O_2 \cdot g^{-1} \cdot h^{-1}$; 31). Although it is tempting to say that tenrecs simply have really low metabolic rates, euthermic (T_b ~36°C) Arctic ground squirrel oxygen consumption rates at T_a 2°C approximate our tenrec active high rates at T_a 12°C and 20°C (32). It is more likely that active tenrecs in the active low state are able to suspend key homeostatic processes and afford a metabolic depression similar to a torpid squirrel, thus allowing for the similarity of oxygen consumption rates in active low and torpid tenrecs. It then follows that tenrecs in the active high state are performing more homeostatic processes and, therefore, experience higher metabolic rates consistent with boreoeutherian rates of oxygen consumption.

If our hypothesis that tenrecs are suspending homeostatic processes when metabolism or T_b is low is true, then one might expect to see increased indications of dysregulation in those states (e.g., significantly altered proteome stability). We investigated this hypothesis by examining whether the liver proteome was markedly affected by T_b and T_a variability. We found that a striking 50.9% of identified liver proteins were differentially abundant between torpid and active animals. In contrast, only 15.5% of identified liver proteins were differentially abundant in Arctic ground squirrels between winter hibernation and summer (33). Protein abundance was highly variable within, but not between warm and cold torpid tenrecs, suggesting that it was significantly altered by hibernation. Of the proteins that increased in active cold relative to active warm tenrecs, 72.2% were also increased

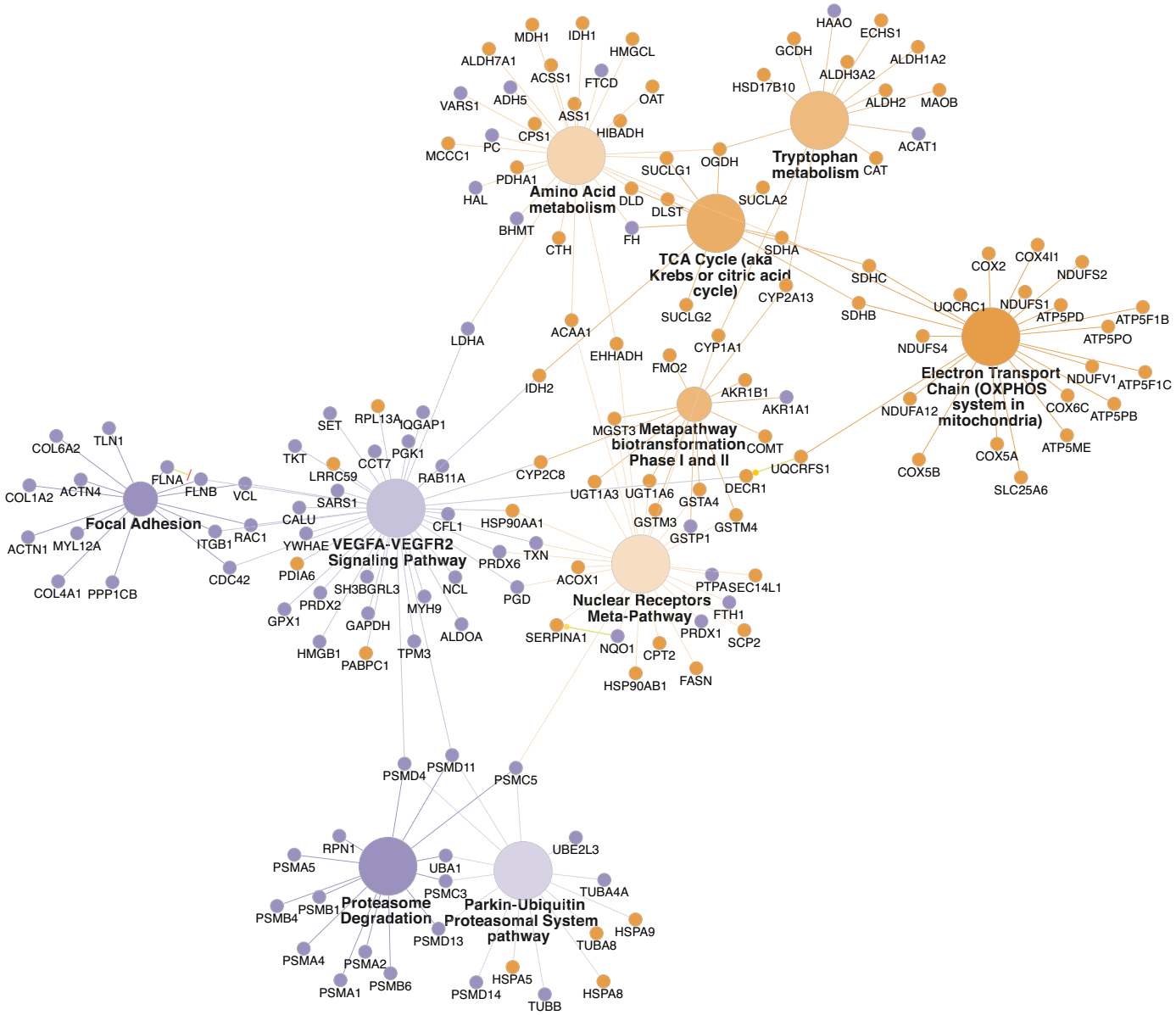

Figure 5. Functionally grouped network of proteins that were differentially abundant between active ($n = 9$ animals) and torpid ($n = 6$ animals) common tenrecs. Functional enrichment was conducted using ClueGO and the human WikiPathways database. The cluster of proteins increased in active relative to torpid animals is shown in orange, whereas the cluster increased in torpid animals is shown in purple. Nodes are shown linked based on their κ score level (≥0.5); node size is scaled by term enrichment significance (adjusted $P < 0.05$) and node opacity is scaled by the proportion of proteins from each cluster associated with the term.

during torpor in warm tenrecs, suggesting that exposure to cold T_a and lowering of T_b during active periods may also perturb the ability to maintain a stable proteome. We also identified nine proteins that were increased in response to cold T_a, regardless of T_b or activity state, which may reflect a general liver response to cold stress. Despite these differences, we found that many of the DAPs in tenrecs were similar to those identified in other hibernating species, from reptiles to primates (34, 35), suggesting that the core elements of metabolic and cellular responses to torpor are highly conserved in phylogenetically disparate mammals.

In ground squirrels, the interbout arousal allows for a resetting of homeostasis wherein transcription, translation, and protein degradation are resumed (e.g., 36). In constrast to other small hibernators, the common tenrec does not experience periodic arousals during hibernation (6, 10). However, that is not to say that all hibernating tenrecs are similar in their torpor extent and duration. In contrast to torpid tenrecs at 12°C, torpid tenrecs at 20°C and even more so at 28°C are usually able to right themselves quickly (6). Furthermore, there is a seasonality to torpor; for example, animals in April are more readily righted than animals in June. We suspect that warmer tenrecs (active or torpid) are more competent to perform homeostatic activities than cold tenrecs. In our colony, cold tenrecs (active or torpid) occasionally experience bleeding from the cloaca, whereas warm

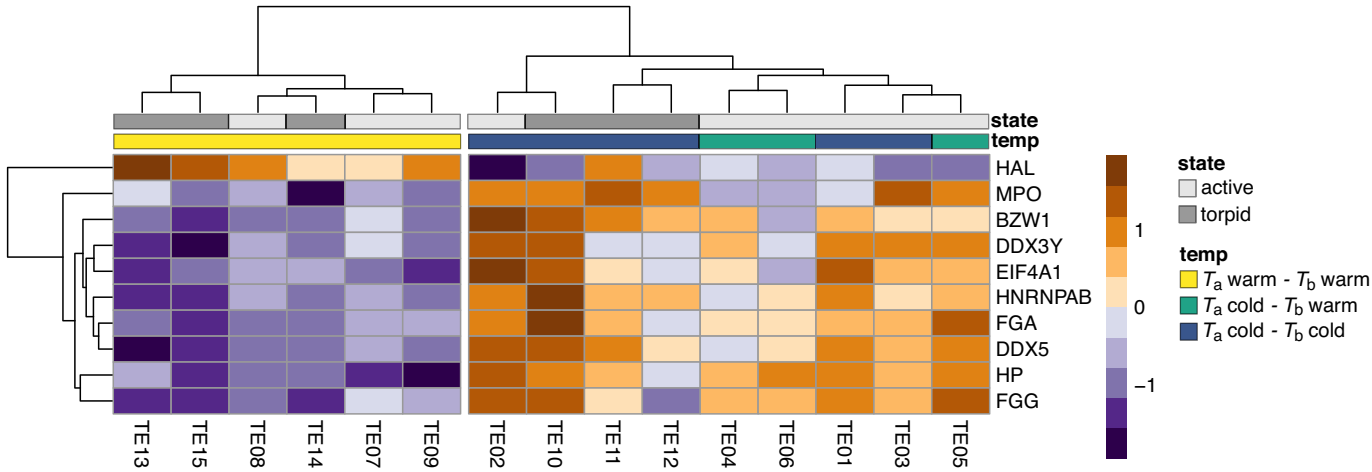

Figure 6. Heatmap showing scaled abundance of 10 proteins that were differentially abundant between common tenrecs at varying ambient and body temperatures (temp; T_a and T_b, respectively), regardless of activity state at time of sampling (state; active and torpid). Rows and columns were clustered by Euclidean distance.

tenrecs seldom do, and in our experience, tenrecs are more likely to spontaneously die during hibernation at lower temperatures (data not shown). During the active season, tenrecs have a very different thermal experience even when maintained at T_a 12°C. Since T_b of active animals waxes and wanes, these tenrecs oftentimes experience warm T_b even though T_a is only 12°C. We speculate that these sojourns to higher T_b may allow for bursts of homeostatic activities much like the interbout arousal of a hibernating ground squirrel. It should be noted that cold temperatures may be a feature of natural tenrec biology. Common tenrecs are found near the high-altitude Andringitra National Park in Madagascar where it may occasionally snow in winter and nighttime low temperatures in summer may be ~12°C (37). It would be interesting to determine whether life history traits like survivorship of hibernation and average age of this natural population are impacted by the low temperatures as our laboratory data might suggest.

The majority of proteins that we identified as differentially abundant between active and torpid tenrecs in this study were associated with protein turnover. Proteins that increased in abundance in torpid tenrec liver included components of the proteasome and proteins associated with the regulation of mRNA processing and protein ubiquitylation, similarly to other hibernating species (11). Although two translation initiation factors (EIF5A and EIF4H) were increased, nine ribosomal proteins were decreased during torpor, consistent with a global depression of protein synthesis observed in other hibernators (38). Depressed protein synthesis should be coordinated by a depression of protein degradation if protein pools are to be maintained for arousal. The cold temperatures of torpor in a ground squirrel markedly depress 26S proteasome activity, but not protein ubiquitylation (39). The result is a two- to threefold increase of ubiquitin-protein conjugates that must be resolved in the interbout arousal. However, torpid tenrecs increased proteasomal components even at warm T_b, suggesting significant protein catabolism during hibernation. We posit this may occur because in contrast to many other hibernators, tenrecs do not typically experience tremendous fattening before hibernation, so they cannot rely on fat stores to fuel metabolism in the absence of eating (personal observation).

One potential mechanism of protein recovery is protection of mRNAs from degradation to enable rapid resumption of protein translation upon arousal, given that a sufficient pool of amino acids remains at the end of hibernation. We found that several mRNA splicing factors were increased in torpid tenrecs, similar to other hibernators, which may contribute to hepatic storage of pre-mRNAs in the splicing and cleavage states during torpor (40). Furthermore, proteins associated with amino acid metabolism made up the largest functional group that decreased in torpid tenrecs and included many enzymes that also decline in abundance in hibernating boreoeutherians (e.g., HIBADH, ALDH7A1; 11). Together, these data suggest that tenrecs may suppress protein translation but not protein degradation during torpor, even at warm T_b, while potentially preserving mRNAs and amino acid pools for arousal. Further studies will be necessary to correlate protein with mRNA transcript abundance and to directly measure rates of protein turnover (e.g., Ref. 41). However, the potential implications of increased protein degradation associated with heterothermy are significant: higher rates of protein turnover have been correlated with reduced life span in mammals (42).

The tenrec proteome also reflected predictable metabolic adjustments to fasting during torpor, such as suppression of oxidative metabolism and increased gluconeogenesis and ketogenesis. A number of enzymes involved in the TCA cycle and ETC were decreased, whereas enzymes associated with glycolysis and gluconeogenesis (e.g., ALDOA), pentose phosphate pathway (e.g., PGD), and metabolism of ketones (e.g., BDH1) and glycogen (e.g., PYGL) were increased in torpid tenrecs, similarly to other hibernators (11). Although one of the hallmarks of hibernation in small mammals is a switch from carbohydrate to lipid metabolism (5), a larger number of enzymes involved in fatty acid oxidation decreased rather than increased in abundance in torpid tenrecs, reflecting an overall suppression in metabolism. Further studies will be necessary to determine the contribution of lipid substrates

Table 1. *Proteins that were differentially abundant in response to both cold exposure and hibernation in common tenrecs*

Known Function
Protein translation, folding, and transport
EEF1B2
EEF1G
EIF4H
EIF5A
NARS
PLIN3
SSR1
RNA transcription, processing, transport, and stability
CIRB
PRBM3*
SND1
DDX39B
SUB1
SRSF2
PTBP1
RANBP1*
Actin binding and regulation
DSTN
LCP1
TMSB4X
TPM3
YWHAZ*
Calcium binding
ANXA2*
S100A10*
AHNAK*
Immune response
CTSB
CTSZ
PTMA

The listed proteins had higher abundance in active cold (T_a cold-T_b cold) tenrecs relative to active warm (T_a warm-T_b warm) tenrecs and in hibernating warm (T_a warm-T_b warm) tenrecs relative to active warm (T_a warm-T_b warm) tenrecs. *Proteins that were also more abundant in hibernating cold (T_a cold-T_b cold) tenrecs compared with active cold (T_a cold-T_b cold) tenrecs. Known functions of human orthologs of tenrec proteins were obtained from UniProt.

to metabolism during torpor (e.g., by metabolomics), as respiratory exchange ratios (moles of CO_2 produced/moles of O_2 consumed) are remarkably variable in tenrecs, suggesting large swings in acid/base balance and precluding predictions on metabolic substrate utilization (6). Proteins associated with synthesis of fatty acids and cholesterol (e.g., FASN, HMGCS1), the urea cycle (e.g., CPS1), and detoxification pathways (e.g., CYP1A1) were decreased in torpid tenrecs, suggesting that these functions are placed on hold during hibernation (11). Although deep hibernators may restore some of these metabolic functions during interbout arousals to maintain metabolic homeostasis at extremely low T_b (4), these pathways may be restricted to active periods of the year in tropical hibernators such as tenrecs.

Other insights from the liver proteome suggest that homeostasis may not be altogether abandoned during torpor in tenrecs. The most highly increased proteins in torpid tenrecs included quinone reductase NQO1, calcium-binding protein S100A10 and its binding partners ANXA2 and AHNAK, and the antiprotease α-2-macroglobulin (A2M). NQO1 plays a significant role in detoxification and production of antioxidants and is upregulated during torpor in bears and bats, potentially as a mechanism to maintain redox homeostasis during hibernation (43). S100A10 and its binding partners have been implicated in regulation of cell cortex architecture and plasma membrane protein organization, activity of calcium channels, and production of plasmin by plasminogen activators (44). Their increase in torpid tenrecs may help to protect liver cells from calcium-dependent proteolysis and/or contribute to fibrinolysis during hibernation. Consistent with the latter hypothesis, the thrombin-inhibiting antiproteinase A2M was also increased in torpid tenrecs, potentially contributing to hypocoagulation during long periods of inactivity (11).

Exposure to cold T_a and lowering of T_b toward T_a caused a shift in the tenrec liver proteome that partially mirrored the response to torpor. Although the highly regulated lowering of T_b in hibernators is considered distinct from hypothermia (4), both physiological states share common protective molecular responses (45). Proteins increased in active cold tenrecs included acute-phase proteins haptoglobin and ceruloplasmin and cold-inducible RNA-stabilizing proteins CIRBP and RBM3. Haptoglobin was the only protein increased in active animals at T_a cold-T_b warm versus T_a warm-T_b warm, suggesting that it may be a marker of cold stress in a tropical mammal. Other proteins that increased in cold tenrecs included DNA helicases, blood-clotting proteins, actin-binding proteins, and factors involved in protein translation, folding, and transport. These data suggest that tenrecs mount a conserved protective response to both hypothermia and torpor that involves maintenance of nucleic acid and cytoskeleton stability and regulation of blood coagulation and calcium homeostasis. However, it is unclear whether this response to torpor at warm temperatures in tenrecs serves to mitigate the effects of prolonged inactivity, hypoxia, T_b-independent changes in metabolic rate, or another stressor.

Boreoeutherians maintain a higher and more stable T_b which should facilitate greater and more predictable coordination of homeostatic processes. This increased homeothermy also allowed for a more effective use of hibernation. Deep hibernators such as ground squirrels exploit the low T_b of torpor to depress homeostatic processes for metabolic savings, for example, translational initiation is uncoupled from elongation as T_b reaches 18°C during entrance into torpor (46). The near-arrest of processes like transcription, translation, and protein degradation means relatively stable protein pools. In contrast, the heterothermy of a tenrec lends itself to poor coordination of homeostatic processes as each enzyme has unique sensitivity to changing temperature. The pronounced heterothermy of the active common tenrec makes an arrest of anabolic or catabolic processes at a particular temperature unlikely. Furthermore, extended periods of low T_b in tenrecs could exacerbate variation as spurious anabolic and catabolic processes occur. The lack of an interbout arousal means that these tenrecs are unable to reset homeostasis like a ground squirrel and more likely to incur mismatches in protein pools when torpid or cold for extended periods of time. We suggest this may be one reason why common tenrec litters are so large (32 confirmed young) as hibernation likely takes a significant toll on populations (6). We also suggest that tenrecs are probably most successful at hibernating in warmer temperatures or where diel oscillations in ambient temperature allow for a sojourn to a warmer

T_b and more homeostatic activities. Finally, we suggest that a more inclusive definition for mammalian hibernation may be the seasonal depression of homeostatic activities below that which is normally required for proper physiological function. The more pronounced homeothermy of boreoeutherians likely led to greater coordination of homeostatic processes and fewer complications due to mismatches in thermal sensitivities of metabolic pathways.

DATA AVAILABILITY

The raw data and MaxQuant output files generated during this study are available at ProteomeXchange MassIVE repository, doi:10.25345/C51J6M (ftp://massive.ucsd.edu/MSV000087062/). The differential protein abundance data generated during this study are included in the Supplemental Data.

SUPPLEMENTAL DATA

Supplemental File 1: https://doi.org/10.6084/m9.figshare.14721315.v1.
Supplemental File 2: https://doi.org/10.6084/m9.figshare.14745255.v1.
Supplemental File 3: https://doi.org/10.6084/m9.figshare.14745270.v1.
Supplemental File 4: https://doi.org/10.6084/m9.figshare.14745273.v1.
Supplemental Code: https://doi.org/10.6084/m9.figshare.14745279.v1.

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DISCLAIMERS

The identification of certain commercial equipment, instruments, software, or materials does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.I.K., M.D.T., and F.v.B. conceived and designed research; J.I.K., M.D.T., M.C.S., J.S.D., and F.v.B. performed experiments; J.I.K., M.C.S., and B.A.N. analyzed data; J.I.K., M.D.T., B.A.N. and F.v.B. interpreted results of experiments; J.I.K. prepared figures; J.I.K. and F.v.B. drafted manuscript; J.I.K., M.D.T., M.C.S., J.S.D., B.A.N., and F.v.B. edited and revised manuscript; J.I.K., M.D.T., M.C.S., J.S.D., B.A.N., and F.v.B. approved final version of manuscript.

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