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**Transcriptomics in the wild: hibernation physiology in free-ranging dwarf lemurs**

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**Running title: Transcriptomics of hibernation in wild dwarf lemurs**

### **Abstract**

Hibernation is an adaptive strategy some mammals use to survive highly seasonal or unpredictable environments. We present the first investigation on the transcriptomics of hibernation in a natural population of primate hibernators: Crossley's dwarf lemurs (*Cheirogaleus crossleyi*). Using capture-mark-recapture techniques to track the same animals over a period of seven months in Madagascar, we used RNA-seq to compare gene expression profiles in white adipose tissue (WAT) during three distinct physiological states. We focus on pathway analysis to assess the biological significance of transcriptional changes in dwarf lemur WAT and, by comparing and contrasting what is known in other model hibernating species, contribute to a broader understanding of genomic contributions of hibernation across Mammalia. The hibernation signature is characterized by a suppression of lipid biosynthesis, pyruvate metabolism and mitochondrial-associated functions, and an accumulation of transcripts encoding ribosomal components and iron-storage proteins. The data support a key role of pyruvate dehydrogenase kinase isoenzyme 4 (*PDK4*) in regulating the shift in fuel economy during periods of severe food deprivation. This pattern of *PDK4* holds true across representative hibernating species from disparate mammalian groups, suggesting that the genetic underpinnings of hibernation may be ancestral to mammals.

**Keywords:** RNA-seq, differential gene expression, *Cheirogaleus*, white adipose tissue

## Introduction

Many species exhibit remarkable phenotypic plasticity in the face of environmental heterogeneity.

One of the most extreme manifestations of this flexibility is seasonal mammalian heterothermy (Boyer & Barnes 1999; Ruf & Geiser 2014; van Breukelen & Martin 2015). Mammals, typically thought of as masters of physiological homeostasis, can abandon constancy in favor of temporally fluctuating body temperatures and depressed metabolism when energetic demands from the environment pose a threat to survival (Carey *et al.* 2003; Geiser 2004; van Breukelen & Martin 2015).

Mammalian heterothermy is expressed in many forms with variations in the degree and length of the response. The commonality among mammalian heterotherms is a constellation of physiological modifications that comprise a phenotype known as torpor. Torpor occurs from a lowering of the thermoregulatory set point in mammals, with some species experiencing set points that allow core body temperatures to drop below freezing (Barnes 1989), while some display more shallow reductions (i.e. a few degrees below euthermic levels as in American black bears; Toien *et al.* 2011). This lowering of body temperature – however extreme or shallow – is driven by a controlled and reversible metabolic depression which amounts to substantial energetic savings (Geiser 2004; Heldmaier *et al.* 2004).

In the original view, seasonal heterothermy was considered to be restricted to temperate and arctic mammalian species solely as an avoidant response to frigid winter temperatures. With the advent of increasingly smaller temperature loggers and more mobile transmitters, however, the field of hibernation research has experienced an explosion of studies investigating small mammals in their natural environments. Together, these studies have revealed that seasonal heterothermy is far more

taxonomically widespread and is used in a much greater variety of ecological circumstances than previously thought. Some of the most compelling and exciting findings arise from the fact that numerous tropical and subtropical species have now been documented to display heterothermic responses that are virtually identical to cold-adapted animals (Boyer & Barnes 1999; McKechnie & Mzilikazi 2011). In response to these findings, a paradigm shift has occurred. In this new paradigm, it is understood that daily torpor and hibernation are not behaviors that function exclusively to avoid energetic costs from cold environmental temperatures, rather, they are more likely deployed as a response to limited resources that present energetic bottlenecks that are especially challenging to small-bodied species (Heldmaier *et al.* 2004; Dausmann *et al.* 2005; Geiser 2013).

The island of Madagascar harbors one of the only groups of primates known to display obligatory hibernation – the dwarf lemurs of the genus *Cheirogaleus* (Dausmann *et al.* 2004; Blanco & Rahalinarivo 2010; Blanco *et al.* 2013; 2016), though other groups have been documented to use torpor opportunistically (Kobbe & Dausmann 2009; Nowack *et al.* 2010). Species in the genus *Cheirogaleus* are nocturnal, small-bodied primates (150-500 g) that experience obligate hibernation during the austral winter. However, unlike cold-adapted hibernating species, dwarf lemur species found in the sub-tropical regions of Madagascar generally experience comparatively higher body temperatures during torpor (~12-15°C; Blanco & Rahalinarivo 2010; Blanco *et al.* 2013; 2016). Thus among the many questions addressed by our study, it remains entirely unknown how hibernating at relatively higher  $T_b$  might be reflected in transcriptomic changes in biologically important tissues, and further, how modifications at the cellular level impact whole-body metabolism in hibernating mammals.

Our study focuses on gene-regulatory patterns expressed in the white adipose tissue (WAT) of dwarf lemurs as extreme fattening is a necessary precondition for prolonged hibernation. It is hypothesized that these primates rely primarily on WAT accumulated in the tail during an intense fattening stage to fuel endogenous energy needs during periods of torpor (Fietz & Ganzhorn 1999; Fietz & Dausmann 2007). We present here a novel investigation of dynamic alterations in gene expression that are correlated with the hibernation phenotype in a species of free-ranging dwarf lemur tracked for seven months in the high-altitude rainforests of central-eastern Madagascar. Using samples collected longitudinally from the same animals during three distinct physiological states, RNA sequencing (RNA-seq) was used to characterize gene expression patterns that correlate with prolonged periods of dormancy and fasting. We focus on pathway analysis by identifying functional groups of co-regulated genes, in addition to the expression of individual genes, to assess the biological significance of transcriptional changes in dwarf lemur WAT.

We predict that the transcriptomic profiles we uncover during three distinct physiological states will identify the biological processes that are important for maintaining functioning during physiological extremes associated with torpor in free-ranging Crossley's dwarf lemurs. The impacts of changes in gene expression in WAT on metabolic profiles throughout the circannual cycle have been investigated in only a few model mammalian species, and always under laboratory conditions (Wilson *et al.* 1992; Herminghuysen *et al.* 1995; Boyer *et al.* 1998; Bauer *et al.* 2001; Demas *et al.* 2002; Buck *et al.* 2002; Eddy 2004; Kabine *et al.* 2004; Eddy *et al.* 2005; Hampton *et al.* 2011), including a captive colony of *C. medius* (Faherty *et al.* 2016b). Thus, to our knowledge, this is the first study of its kind to focus on the longitudinal transcriptomic changes that drive hibernation physiology in free-ranging animals under natural conditions, especially in primate heterotherms. Our results show broad implications for understanding the evolutionary dynamics of hibernation in

mammals generally, in addition to revealing patterns of genotype to phenotype interactions in natural environments that appear to be universal to mammalian hibernators.

## Materials and methods

**Overview.** We conducted three field seasons during 2013 that coincided with distinct physiological states dwarf lemurs experience during their circannual cycle: March (Fattening), July (Torpor), and September (Emergence, within 1-2 days of exiting hibernacula; Figure 1). During a torpor bout, wild dwarf lemurs exhibit lowered  $T_b$  and metabolic rate, reductions in heart rate to 3-5 beats per minute, and episodes of irregular and infrequent breathing (Blanco & Rahalinarivo 2010; Blanco *et al.* 2013).

**Study area.** Animals used during this study were captured at Andasivodihazo, a forest fragment in Tsinjoarivo Forest (~225 ha; 19° 41'15" S, 47°46' 25" E). This forest is one of the last remaining high-altitude rainforests (1600-1700 m) in central-eastern Madagascar. Climate in Tsinjoarivo is one of the coldest environments in Madagascar and is characterized by a distinct rainy season (December-March) followed by a dry season (April-November), with average annual rainfall recorded at 2000 mm (Blanco & Rahalinarivo 2010). At Tsinjoarivo, highest  $T_a$  are recorded during the austral summer (December-January), but due to high elevation, temperatures never exceed 30°C (Blanco & Rahalinarivo 2010; Blanco *et al.* 2013). Maximum  $T_a$  during the midpoint of the hibernation season (June/July) averages 19°C, but consistently dips to around 5°C overnight, with below freezing temperatures on rare occasions.

**Study animals.** A total of six Crossley's dwarf lemurs (*C. crossleyi*; 4 females and 2 males) were monitored and used for sample extraction. All animal procedures were completed in the field under natural conditions. The study was done in accordance with the current laws of Madagascar using permits issued under the Ministère de l'Environnement et des Forêts. Animal protocols complied with those of Duke University's Institutional Animal Care and Use Committee (IACUC #A017-15-01).

**Capture/mark/recapture techniques.** Animals were live-trapped using Tomahawk traps (5" x 5" x 16"), baited with fermented banana and set between 4 -10 m high at established trapping locations in known home ranges. Captured individuals were sexed, measured, weighed, and individually marked with microchips (AVID Identification Systems, Inc., CA, USA).

In March, six animals were equipped with external radio transmitters (radio-collars) to assist in locating and recapturing them during the study period. Collars were placed on the animals when they were about to enter hibernation, and were thus at their heaviest (i.e. thickest neck circumference), and were removed shortly after the animals emerged from hibernation in September. In July, animals were re-captured and the fit of the collars was checked to ensure safety.

Transmitters' signals were checked daily in March and September to determine locations of sleeping sites, and weekly in July to localize underground hibernacula using telemetry equipment (Receiver R410, Advance Telemetry System, Isanti, MN) and a 3-element Yagi antenna. During the hibernation season, collared dwarf lemurs were found underground, between 10-40 cm deep. Individuals were removed from their natural hibernacula for tissue sampling and morphometric measurements and were released to the same locations after dark, while they were alert. They were checked daily, post-release, to ensure they returned to hibernation.

**Temperature measurements.** Radio-collars were equipped with a sensor to record skin temperature every 60 minutes for the entire study period (ARC 400, 10 g, Advanced Telemetry Systems, Isanti, MN) (Figure 1). The collar size/body mass ratio was < 4%, well within the accepted range for mammals. Previous studies have demonstrated that skin temperature recorded from temperature-sensitive collars accurately reflects body temperature when individuals are curled up during hibernation (Dausmann 2005; Munro *et al.* 2005). Hibernacula temperatures ( $T_{hib}$ ) were recorded by data loggers (Maxim DS1922 iButton, Maxim Integrated Products, Inc., San Jose, CA) placed in the soil near the animal, about 5 cm under the surface. A logger placed in a shady area in the forest recorded ambient temperature ( $T_a$ ) hourly.

**Tissue collection and RNA extraction.** Physiological state at each collection point was verified by rectal  $T_b$  and confirmed using data recovered from collars (Table 1; Table S1; Figure 1). We used minimally invasive sampling techniques to acquire tissue samples of WAT via biopsy, as previously described (Faherty *et al.* 2016b; see also Supporting Information). Tissue samples of WAT were obtained from the base of the tail where fat storage was most concentrated. Biopsy was removed and tissue sample was expelled directly into RNA stabilization solution (Qiagen; Valencia, CA). Samples were stored at  $T_a$  until importation into the US. The entire tissue sample (15-20 mg) was used for RNA isolation procedure.

Due to working with captive endangered primates, such as dwarf lemurs, certain restrictions are posed on amount of tissue we were able to obtain. In addition, WAT has the added complication of having a high lipid content to nuclear content ratio and extracting sufficient total RNA for downstream RNA sequencing is problematic. To that end, we completed a whole transcriptome amplification step on all total RNA extractions prior to Illumina sequencing, using NuGEN's Ovation

RNA-Seq V2 kit (San Carlos, CA) according to manufacturer's instructions (exact protocol can be found in the Supporting Information). In a preliminary analysis using unamplified vs. amplified RNA extracted from rat WAT, we have confirmed that whole transcriptome amplification does not introduce significant bias in relative mRNA frequencies (Faherty et al., 2015). Amplified cDNA samples from dwarf lemurs were then sent to Duke University's Genome Sequencing Shared Resource for library preparation and sequencing.

**Library preparation and Illumina sequencing.** Previously amplified cDNA libraries were prepared for sequencing using Illumina's TruSeq DNA Sample Preparation Kit. Final library size distribution was determined using Agilent Bioanalyzer 2100 and insert sizes were 300 base pairs (bp), as per standard library prep. Libraries were pooled and sequenced on two lanes of the Illumina HiSeq2000 platform (San Diego, CA) using the rapid-run mode with 150 bp paired-end reads. The content of each library was divided by half and each half sequenced on one of the two lanes. This was done to avoid lane-related batch effects. Library preparation and Illumina sequencing was performed at the Duke University's Sequencing and Genomic Technologies Shared Resource. Raw sequence data were deposited into the NCBI Short Read Archive with accession number PRJNA400868 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA400868/>).

**Quality control and filtering of sequencing reads.** Quality control inspection of raw sequence data was done using FastQC and filtering was completed using the software Trimmomatic (Bolger *et al.* 2014). Trimmomatic removed the Illumina adaptor sequences and trimmed the ends of the reads by checking the first and last three bases and retaining reads with an average Phred score >15 (SLIDINGWINDOW:4:15), with a required minimum length of 100 bp.

**De novo transcriptome assembly.** *De novo* assembly of the WAT reference transcriptome was performed using the software Trinity (v. 2.1.0; Haas *et al.* 2013), pooling together reads from all samples (n = 17, see Supporting Information). We used *in silico* read normalization and required a minimum length of 400 nucleotides to minimize artifactual transcripts. Trinity was run on a machine with 48 cores and 512 Gb of RAM. Final assemblies have been uploaded to Dryad (doi:10.5061/dryad.h8f66).

To measure the quality of the assembly we used the Transrate software (Smith-Unna *et al.* 2016). This software uses features such as consistent and accurate read mapping, and homology against proteins from a related species (*Homo sapiens*) to generate a global score for a given assembly. It also generates a reliability score for each transcript. We kept only transcripts with a Transrate score above 0.36 (994,248 transcripts). To annotate our assembly, we ran sequence similarity searches using BLASTX against the UniProt database (release date: 2015-03) and against the human proteome database (Ensembl v.83). Only transcripts with detectable homology to the human proteome (BLASTX E-value < 10<sup>-4</sup>) were used for downstream analyses. When several transcripts mapped to the same human protein, the one that reconstructed a larger portion in relation to the human protein length was kept. Subsequently, we selected transcripts that showed significant sequence similarity to at least 70% of the human protein sequence length. The final dataset contained 15,768 transcripts with homology to human proteins.

#### **Quantification of transcript abundance, differential expression, and functional enrichment**

**analysis.** To perform differential gene expression analysis, we used sequencing data from 4 of the 6 individuals (MA, DA, NA and BL; see Supporting Information; Table S1). One of the previously monitored individuals (AN) could not be recovered at emergence and samples from this animal were

discarded to ensure consistency. The second discarded individual (NE) had anomalous high weight at the hibernation collection point and was thus also considered not sufficiently reliable.

The software Kallisto (Bray *et al.* 2016) was used to quantify the expression levels of transcripts in each sample from the common reference transcriptome using pseudo-alignments. This strategy calculates the potential loci of origin along the reference transcriptome and uses less computational resources than traditional read mapping. We used a bootstrap value of 25 (-b) to measure technical variance in the abundance estimates. We next transformed the table of counts (number of reads per gene) to counts per million in logarithmic scale (log cpm) with EdgeR (Robinson *et al.* 2012; McCarthy *et al.* 2012) and applied multi-dimensional scaling (MDS) to visualize the level of similarity at the level of gene expression of the different sample points.

The quantification and bootstrap values were fed into Sleuth to generate lists of differentially expressed genes in each pair-wise comparison (Pimentel *et al.* 2016). We only tested for differential expression transcripts with at least 5 reads in half of the samples ( $n = 10,745$ ). We performed pairwise comparisons between the three physiological conditions and retrieved the genes with an adjusted p-value  $< 0.05$ .

Functional enrichment clustering of the differentially expressed transcripts was conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; Huang *et al.* 2007). This software uses a combination of sequence features, functional annotations and pathway information to identify overrepresented functional gene clusters in a set of sequences. We selected clusters with an enrichment score  $> 1.3$  (equivalent to a non-log scale 0.05; Haung *et al.* 2009) and containing significantly overrepresented terms at an adjusted p-value  $< 0.05$  using Benjamini-Hochberg correction.

## Results

**Data collection.** During three field seasons at Tsinjoarivo Forest, Madagascar, we sampled tail white adipose tissue from six individuals during three time points: Fattening, Torpor, and Emergence. Animal weight at Emergence, 1-2 days after emerging from Torpor, was about 65% of the weight measured during Fattening (Table 1). RNA extracted from white adipose tissue (WAT) was subjected to high-throughput RNA sequencing (RNA-seq) to identify gene expression profiles that are correlated with changes in physiology. A total of 17 libraries were sequenced (6 animals x 3 time points for each, with the exception of individual AN who was missing a sample collected from the Emergence time point).

Using Trinity, we generated a reference *de novo* transcriptome assembly for *C. crossleyi* WAT. After several quality filtering steps (Table 2; see Methods for more details) we kept 15,768 unique transcripts that showed extensive sequence similarity to human proteins and which could thus be unambiguously annotated.

**Quantification of transcript abundance for the three collection points.** Using multi-dimensional scaling (MDS) to visualize the level of similarity at the level of gene expression of the different sample points, we found that samples from each of the three conditions – Fattening, Torpor, and Emergence – clustered together, as expected (Figure 2; see also Figure S1 with all six initial individuals included). We tested for differential gene expression between all pairs of conditions using the program Sleuth (Pimentel *et al.* 2016). We found that 377 genes are differentially expressed when comparing Fattening vs. Torpor, 3 genes showed variable expression during the Torpor vs. Emergence comparison, and 220 genes were differentially expressed when comparing Fattening vs. Emergence (Table 3; Table S2). The low number of differentially expressed genes between Torpor and Emergence indicated that most Torpor-related gene expression changes have

not been yet completely reverted when the samples were collected at Emergence. This was in agreement with the intermediate position of the Emergence samples when general differences in gene expression were assessed by multidimensional scaling (Figure 2). However, this finding may also indicate that there may be a biological difference in global gene expression between dwarf lemurs, which engage in torpor in a relatively warmer environment than those species studied to date which present body temperatures near freezing.

Not surprisingly, there was a high number of differentially expressed genes that overlapped in the comparisons Torpor vs. Fattening and Emergence vs. Fattening ( $n = 86$ ), about one order of magnitude higher than that expected by chance. Among these genes, 43 genes were upregulated, and 43 genes were downregulated in Fattening versus the other two conditions. We investigated the enrichment of certain functional classes in the set differentially expressed genes using DAVID (Huang *et al.* 2007), focusing on genes that showed significant differences between Fattening and Torpor (Figure 3). The distribution of gene expression values of a selected subset of genes is shown in Figure 4. The lack of overlap in the gene expression values between Fattening and Torpor indicates very consistent changes between these two conditions.

**Metabolic switch from carbohydrates to lipids.** Diverse studies have documented a switch from a carbohydrate to a lipid-based metabolism during bouts of torpor (Carey *et al.* 2003). Experiments using 13-lined ground squirrels (*Ictidomys tridecemlineatus*) have shown that this is associated with increased levels of pyruvate dehydrogenase kinase isoenzyme 4 (*PK4*). This enzyme inhibits carbohydrate catabolism by preventing the conversion of pyruvate to acetyl-CoA (Buck *et al.* 2002). Shallow torpor under laboratory conditions also induces *PK4*, in agreement with a previous study conducted at the Duke Lemur Center (Durham, NC, USA) with dwarf lemurs of the species *Cheirogaleus medius* (fat-tailed dwarf lemurs; Faherty *et al.* 2016b). The present study provided a unique opportunity to investigate whether similar changes occurred in dwarf lemurs undergoing torpor in the wild.

We observed that *PDK4* was also strongly induced during Torpor in wild dwarf lemurs (between 10-50-fold; Figure 4), whereas the gene products of all three enzymes involved in the pyruvate dehydrogenase complex were down-regulated during Torpor; two subunits of pyruvate dehydrogenase (lipoamide) alpha 1 (*PDHA1*) and pyruvate dehydrogenase (lipoamide) beta (*PDHB*), dihydrolipoamide S-acetyltransferase (*DLAT*) and dihydrolipoamide dehydrogenase (*DLD*) (Figure 4). In contrast, lipid catabolism was clearly increased during Torpor. This was reflected in a significant enrichment in phospholipid phosphatase 1 (*PLPP1*) and apolipoprotein C-II (*APOC2*), and a decrease in the expression of genes involved in lipid biosynthesis, including stearoyl-CoA desaturase (*SCD*), fatty acid synthase (*FASN*), and ELOVL fatty acid elongase 6 (*ELOVL6*; Figure 4). These results supported the metabolic switch from carbohydrates to lipids during Torpor in free-ranging *C. crossleyi*.

**Mitochondrial function.** An inhibition of respiration has been observed in several hibernators (Carey *et al.* 2003). We observed an underrepresentation of mitochondrial genes during Torpor in contrast to Fattening, consistent with a general depression in mitochondrial function and oxygen consumption (Figure 3). Peroxisome proteins were also underrepresented; these organelles have a function in the beta-oxidation of very long chain fatty acids in conjunction with the mitochondrion.

**Iron-storage proteins.** Interestingly we also found that two transcripts involved in iron storage and release – ferritin, light polypeptide (*FTL*), and ferritin, heavy polypeptide (*FTH1*) – are very highly expressed during Torpor relative to the Fattening state. Up-regulation of these proteins was between 2- to 8-fold depending on the individual (Figure 4). The genes are expressed at very high levels in normal conditions, so any increase implies an important energy expense. The increase in ferritin may be a response against oxidative stress.

**Translation-related functions.** Surprisingly, the Torpor time point also showed enrichment in pathways that are related to protein translation. This included ribosomal proteins, but also proteins involved in rRNA or mRNA processing. It is yet unclear whether this is a genuine response to Torpor conditions, or a result of increased stability of these transcripts compared to others in the cell.

## **Discussion**

Our study investigated changes in transcriptomic profiles during three distinct physiological states in order to identify biological processes that are important for maintaining functioning during physiological extremes associated with torpor in free-ranging Crossley's dwarf lemurs. Our collection points were selected to capture dramatic changes in physiology. As hypothesized, we expected to see concomitant changes in gene expression correlated with circannual modifications in physiology. Dwarf lemurs sampled during autumnal Fattening were engaged in an intense period of nightly feeding in preparation for the winter fast, while animals sampled in the Torpor time point had been engaged in at least 2.5 months of continuous hibernation. Lastly, all study animals we re-captured within 1-2 days of emerging from hibernation in spring, thus we should expect normal feeding and cellular function to have fully resumed (Boyer & Barnes 1999; Carey *et al.* 2003).

### **Genes involved in protein translation are up-regulated in hibernating dwarf lemurs.**

Results from this study indicate that transcripts related to translation (e.g. ribosomal proteins, rRNA processing, RNA binding) are more abundant during Torpor than either Fattening or Emergence physiological states in dwarf lemur WAT. A result such as this could arise from two scenarios. In scenario one, this result suggests there is a recruitment of protein biosynthesis machinery during torpor to actively engage translation. Conversely,

scenario two would suggest that our findings are resultant from an increase of mRNA stability of translation-related transcripts during torpor bouts.

Similar to dwarf lemurs, it has been shown that the coordinated up-regulation of protein biosynthesis genes are a distinctive feature of the transcriptome profile in heart, liver, and skeletal muscle in hibernating American black bears (*Ursus americanus*; Fedorov *et al.* 2009; 2011; Fedorov 2014), but not in bone tissue (Fedorov *et al.* 2012). The authors of this work hypothesize that up-regulation of protein anabolism pathways contributes to the ability to reduce muscle atrophy over prolonged periods of immobility during hibernation (Fedorov *et al.* 2009). In contrast, the literature is replete with instances of conflicting reports regarding patterns of protein translation during hibernation in ground squirrel species – another group of model mammalian hibernators (*Ictidomys* spp.). Results from transcriptomic and proteomic screens in several tissues reveals both an up-regulation of protein synthesis (Epperson 2004; Epperson *et al.* 2010; Schwartz *et al.* 2013; Fedorov 2014), and a down-regulation of protein synthesis (Yan 2006; Schwartz *et al.* 2013) during torpor. Further, additional studies involving hibernating greater horseshoe bats (*Rhinolophus ferrumequinum*) suggest that protein biosynthesis is suppressed in brain tissue (Lei *et al.* 2014), and in liver and muscle of big brown bats (*Eptesicus fuscus*; Yacoe 1983). This ambiguous relationship is likely due to the inconsistent sampling strategies and experimental methodology across studies. Sampling time points ranged from deep torpor, to entrance into torpor, to just preceding an IBA, which may be reflecting incongruous results. van Breukelen and Martin (2001) find that initiation of protein synthesis is suppressed at 18°C – a result indicative of both temperature-dependent function that you would expect to find during the lowered body temperatures exhibited during torpor, as well

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as an active suppression (van Breukelen & Martin 2001). This finding has been confirmed by others (Gulevsky *et al.* 1992; Chen *et al.* 2001).

An up-regulation of pathways involved in protein biosynthesis in dwarf lemurs – an energetically expensive process – would be generally surprising as, during the hibernation season, dwarf lemurs cease feeding for up to five months and display whole-body metabolic suppression to conserve the limited energy stores in tail fat (Blanco & Rahalinarivo 2010; Blanco *et al.* 2013; 2016; Faherty *et al.* 2016b). If scenario one were indeed the case, the energy cost associated with increased protein biogenesis in WAT may be an important trade-off to allow dwarf lemurs to maintain body condition throughout the hibernation season, thus promoting positive effects on survival through enhanced means to reproduce, support offspring via lactation, and avoid predators after emergence. However, our study design makes it impossible to test this hypothesis as we were only measuring levels of steady-state transcription. Therefore, the more conservative hypothesis is that the second scenario – stability of mRNA transcripts – is a much more likely explanation for increased transcript abundance during Torpor as opposed to new transcription of protein related genes. Knight *et. al* (2000) conducted a study investigating this phenomenon in Arctic ground squirrels (*I. parryii*). The authors examined the poly(A) tail lengths of liver mRNA during different physiological states throughout the year. They found that poly(A) tail lengths were conserved during torpor, suggesting that mRNA is remarkably stable throughout a torpor bout (Knight *et al.* 2000). Their results were consistent with other studies which investigated the stability of a specific mRNA, *GAPDH*, using Northern blot analysis (Frerichs *et al.* 1998; O’Hara *et al.* 1999). This is clearly an avenue that warrants

further study in our system, and others, via nuclear run-on assays or proteomic screens of these genes during torpor bouts.

**Dwarf lemurs demonstrate functional enrichment lipid biosynthesis pathways during**

**Fattening.** Our results also indicate a prominent signature of enriched pathways for increased lipid biosynthesis pathways during Fattening relative to Torpor, a result we expected to find. The findings at the gene expression level indeed correlate nicely with what can be seen from morphological changes alone. Dwarf lemurs nearly double in body mass as they are preparing for the hibernation season via excess fat reserves in the tail where our samples were collected. In other hibernating species (e.g. American and Japanese black bears; *Ursus* spp.; Fedorov *et al.* 2011; Shimozuru *et al.* 2012), thirteen-lined ground squirrels (*I. tridecemlineatus*; Hampton *et al.* 2011) and arctic ground squirrels (Yan 2006; Williams *et al.* 2011; Xu *et al.* 2013) and certainly in captive *C. medius* at the Duke Lemur Center (Faherty *et al.* 2016b), genetic pathways involved in lipid biosynthesis are also up-regulated during the transition into the hibernation season.

Taking a closer look at some of the representative genes in lipid biosynthesis pathways allows us to tease apart some of the more biologically meaningful actions of these individual genes. For example, one gene, *SCD* – a key enzyme in fatty acid biosynthesis – catalyzes the rate-limiting step in the formation of monounsaturated fatty acids (Paton & Ntambi 2009). This gene is also up-regulated in preparation for hibernation in Arctic ground squirrels (*I. parryii*; Yan 2006; Williams *et al.* 2011; Xu *et al.* 2013). It functions by forming a double-bond in stearoyl-CoA to convert saturated fatty acids into monounsaturated fatty acids (Paton & Ntambi 2009). Monounsaturated fatty acids are the substrates for the synthesis of a variety of lipids, such as phospholipids and triglycerides – the storage form of excess fat stored in dwarf lemur tails. Our results also suggest that *FASN*, a complex

that synthesizes long-chain saturated fatty acids from acetyl-CoA, malonyl-CoA and NADPH (Jayakumar *et al.* 1995), is up-regulated in Fattening in relation to Torpor. Additionally, another gene, *ELOVL6* which catalyzes the first and rate-limiting reaction that make up the long-chain fatty acids elongation cycle (Matsuzaka *et al.* 2007; Ohno *et al.* 2010), is also up-regulated in concert with *SCD* and *FASN*. A similar trend regarding *ELOVL6* has been shown in Arctic ground squirrels (*I. parryii*; Yan 2006). It is suggested that *ELOVL6* has a propensity to elongate saturated fatty acids as opposed to unsaturated fatty acids (Matsuzaka *et al.* 2007). Previous work on the physiology of fat stores in the context of hibernation, postulates that the ratio of unsaturated and saturated fatty acids can impact the fluidity of fat stores at lower temperatures. Fat reserves with more unsaturated fatty acids have a lower melting temperature than typical mammalian fat of around 30°C (Florant 1998; Faherty *et al.* 2016a). For hibernation dwarf lemurs, this is critical as body temperatures are around 15°C and fat reserves, in the absence of feeding, would need to remain fluid for accessibility of metabolic substrates (Florant 1998). Fietz *et al.* (2003) investigated the fatty acid composition of fat stores before and during hibernation in the western species of dwarf lemurs (*C. medius*), finding that monounsaturated fatty acids – synthesized from high-sugar fruits ate during pre-hibernation fattening – were the main fuel source in this species during torpor bouts (Fietz *et al.* 2003). An interesting follow-up investigation in our study system could explore which type of fatty acid, saturated or unsaturated, is preferentially synthesized as the animals prepare for hibernation, and concomitantly utilized during torpor bouts.

**The pyruvate dehydrogenase complex is down-regulated during Torpor as compared to Fattening.** The results from the study presented here indicate that the pyruvate dehydrogenase (PDH) complex is suppressed during torpor bouts. The PDH complex is a comprised of three enzymes that convert pyruvate into acetyl-CoA, thereby controlling aerobic oxidation of carbohydrates in the mitochondrial tricarboxylate acid (TCA) cycle

(Young *et al.* 1998). Our results show that all three enzymes, *PDHA1/PDHB* (both subunits of pyruvate dehydrogenase), *DLAT*, and *DLD* are down-regulated during Torpor as compared to Fattening. As metabolic readjustment is necessary to survive hibernation, our results suggest that the down-regulation of the PDH complex assists in regulating the glycolytic intermediates into the TCA cycle thereby decreasing glucose utilization and shifting metabolism to lipids during torpor bouts. Additionally, we also find that *PDK4*, a regulator of the PDH complex, shows higher levels of expression during Torpor than either Fattening or Emergence. This enzyme, when expressed, phosphorylates and inactivates the PDH complex (Andrews *et al.* 1998; Buck *et al.* 2002). The upregulation of *PDK4* provides a molecular mechanism that accounts for suppression of genes involved in the PDH complex in WAT. These patterns of expression regarding the PDH complex have been seen in multiple tissues in ground squirrel species (Andrews *et al.* 1998; Buck *et al.* 2002; Wijenayake *et al.* 2017) and American black bears (Fedorov *et al.* 2011).

**Dwarf lemurs invest much energy into up-regulation of iron storage genes during Torpor.**

Interestingly, we find very high levels of expression in the iron storage genes, *FTH1* and *FTL*. Both genes are part of the complex, ferritin, which acts to store iron in a soluble, non-toxic and readily available form (Orino *et al.* 2001). The increased expression of these genes suggests a key role for ferritin in dwarf lemurs undergoing torpor and as they transition to the active state when rewarming, as one of the genes *FTH1* was also highly expressed during Emergence. Biggar *et al.* (2015) also find *FTH1* to show elevated expression in torpid mouse lemurs (*Microcebus murinus*) – a closely related lemur species that uses torpor opportunistically. They suggest that iron storage is a mechanism the animals are using for protection from iron catalyzed oxidative damage (Biggar *et al.* 2015). However, it may also be that the animals are sequestering iron to protect it during torpor, however, the current study presented cannot differentiate the two possible explanations. In a previous study with a captive colony of dwarf lemurs we found another iron-related gene,

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haptoglobin (*HP*) to show extreme levels of expression during Torpor relative to an active state (fold change of 7.22; Faherty *et al.* 2016b). This gene was also shown to be highly expressed during Torpor in other hibernating species (Mominoki 1998; Mominoki *et al.* 2005; Yan 2006; Chow *et al.* 2013; Vermillion *et al.* 2015).

**The limitations of working with endangered primates under field conditions.** This study presents the first investigation into the genomic mechanisms of primate hibernation in the wild; however, it is not without its limitations and weaknesses, which warrant mention. Capturing wild-caught animals and recovering them certainly presents a challenge in any longitudinal field study and the presented study here is no exception. Our sample sizes are relatively small, only 6 animals could be tracked before and during hibernation, and one of them could not be recovered at emergence. Ideally, studies using RNA-seq should be performed with large sample sizes due to the intrinsic high variability in gene expression across individuals. Additionally, our samples were unbalanced in terms of gender. We sampled two males and four females, and removed one male and one female each from our gene expression analysis. In an ideal study, gender would be balanced as a means to neutralize any gender effects. Alternatively, a researcher may focus on one gender alone; yet, field studies do not present such a luxury as researchers are limited by what animals they can initially trap and collar.

As this study was completed with free-ranging animals, we could not control for animal diets during the Fattening and Emergence time points, time of day during sampling, and length of time between uncovering the animals from their underground burrows and sampling. This presents another variable that is challenging to fit into our analysis: How much does animal handling previous to sampling influence the global transcriptome? Although, the body temperature recorded at the time

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of sampling suggested the animals were deep in torpor, this does not necessarily mean that cellular reactions (e.g. RNA transcription and translation) were not resuming to euthermic levels. Previous studies have documented arousal from torpor to occur rather quickly. Ground squirrels, for example, can rewarm from near freezing body temperatures to euthermic levels in 2-3 hours (Utz and van Breukelen, 2013). Other studies have shown that rewarming rates for golden-mantled ground squirrels (*Callospermophilus lateralis*) can be nearly 1.5°C per min (Utz *et al.* 2007). To our knowledge, no studies have been done which investigates how rates of rewarming impact gene expression. We did our best to keep animal handling to a minimum and keep the time of uncovering the animal to sampling to five minutes or less, but the above question remains. Another drawback to our study was that we were not investigating protein expression – only transcript levels. However, previous studies suggest that protein and gene expression levels are generally tightly correlated in a variety of different tissues and cell types (Kosti *et al.* 2016). A further avenue for future research would be to test both protein and mRNA levels in our study system, as well as confirm gene expression levels using qPCR, though small tissue sample amounts and small sample sizes precluded us from doing so in the present study.

**The power of studying dwarf lemur behavior and physiology under entirely natural conditions.**

This study demonstrates why some studies, such as those on hibernation physiology, are best conducted in the field under entirely natural conditions, at least in critically endangered lemur species. Although acknowledging the difficulty of tracking animals, variables that cannot be controlled as well as under laboratory settings, and overall challenging working conditions, our field study uncovered more genes that were found to be differentially expressed when comparing our former captive study with dwarf lemurs at the Duke Lemur Center (Faherty *et al.* 2016b). In both studies, differential expression was investigated using four individuals. We found very few genes that were common among both experiments: 7 genes (*PK4, RNF125, SOD2, RDH10, FGFR1,*

*ATP1A1*, and *ANGPTL4*) up-regulated during the Torpor time point and 6 genes (*RARRES1*, *NDUFC2*, *RFTN2*, *TMEM135*, *PCMTD1*, and *GSTA3*) down-regulated during Torpor in both experiments. The captive conditions under which that study was conducted may have biased our results and therefore those results should be interpreted with caution. For example, while animals were in a fasted state at time of sampling during torpor, animal regulatory boards require that captive animals be fed throughout the hibernation season, despite an obvious deviation from normal ecological conditions of these animals; and thus, we were only able to investigate a partial response to torpor. However, it should be acknowledged here that most studies done on hibernation in captivity in ground squirrel species, for example, very closely monitor natural conditions and therefore those results are likely very similar to what is seen under natural conditions. The present study demonstrates that a complete investigation of the genomic changes underlying hibernation physiology in dwarf lemurs has not been complete until animals that engage in torpor are studied in their natural environments.

This study was the first to analyze differential gene expression in the field under entirely natural conditions. Further we compare results to multiple evolutionarily-distant species, including ground squirrels, black bears and bats, and a sister lineage of captive primate hibernators in an effort to identify common gene expression profiles underlying the hibernation phenotype. This study uncovers a shift in fuel economy in a natural population of Crossley's dwarf lemurs as well as increased mRNA stability in transcripts related to protein translation during bouts of torpor. The comparative transcriptome analyses reported here generate future studies that include validation of transcriptomic changes of genes involved in protein translation, lipid synthesis, and pyruvate metabolism using proteomic approaches. Further, follow-up studies – including sampling from different time points throughout the hibernation season (e.g. entrance into torpor, early and late torpor) – would allow us to fully capture the metabolic heterogeneity that occurs during seasonal heterothermy in free-ranging dwarf lemurs.

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**Data Accessibility.** Raw sequence data were deposited into the NCBI Short Read Archive with accession number PRJNA400868 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA400868/>) and final transcriptome assemblies have been deposited into Dryad ([doi:10.5061/dryad.h8f66](https://doi.org/10.5061/dryad.h8f66)).

**Authors' contributions.** SLF designed the study, conducted fieldwork and the lab component, and led the writing. JLVC optimized and performed the bioinformatics analyses, conducted data analysis, and generated figures. MBB performed the field experiment and assisted with sample importation. MMA contributed to the design of the bioinformatics pipeline and conducted data analysis, and ADY supervised and partially funded the project. All authors contributed to the writing and approved the final version of the manuscript.

**Table 1.** Physiological and environmental parameters during sample collection. Data is from six Crossley's dwarf lemur individuals.  $T_b$  = body temperature at time of sampling. Euthermic  $T_b$  of Crossley's dwarf lemurs is  $\sim 36^\circ\text{C}$ .  $T_a$  = ambient temperature. Number was calculated from all hourly recordings for the month of sampling, as sampling procedures took place on different days. Recordings from September only go until 11 September, as loggers were removed.  $T_{hib}$  = hibernacula temperature during the month of July.

Field Season	Physiological state	Body mass ( $\pm$ SD; g)	$T_b$ ( $\pm$ SD; $^\circ\text{C}$ )	$T_a$ ( $\pm$ SD; $^\circ\text{C}$ )	$T_{hib}$ ( $\pm$ SD; $^\circ\text{C}$ )
March 2013	Fattening	410.0 $\pm$ 79.1	euthermic	18.9 $\pm$ 2.5	--
July 2013	Torpor	380.3 $\pm$ 50.6	15.9 $\pm$ 2.0	12.5 $\pm$ 2.9	11.4 $\pm$ 0.8
September 2013	Emergence	265.5 $\pm$ 42.2	euthermic	14.8 $\pm$ 4.1	--

**Table 2.** Summary statistics of the sequencing runs and transcriptome assemblies

<hr/>	
<b>Category</b>	
<hr/>	
Raw sequencing reads	393,053,966
Reads retained post-filtering	325,772,855
Percentage of reads retained	82.9%
N assembled contigs	1,863,455
Mean length of contigs	951
N contigs > 1,000 bp	543,950
N contigs > 10,000 bp	331
N50	1076
% contigs mapped to final reference	75.6%
# of unique human proteins	15,768
# of genes tested for DE	10,745

**Table 3.** Differentially expressed (DE) genes analyzed during each sample collection point using Sleuth with a false discovery rate < 5%. The number of up-regulated and down-regulated genes are referring to the first physiological state listed in the pair-wise comparison; DE = differentially expressed

<b>Pair-wise comparison</b>	<b>Number of DE genes</b>	<b>Up-regulated genes</b>	<b>Down-regulated genes</b>
Torpor vs. Fattening	377	189 (50.1%)	188 (49.9%)
Emergence vs. Torpor	3	0 (0%)	3 (100%)
Emergence vs. Fattening	220	106 (48.2%)	114 (51.8%)





