Received: 2 May 2018 | Revised: 5 September 2018 | Accepted: 7 September 2018

DOI: 10.1111/mec.14876

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

A functional transcriptomic analysis in the relict marsupial *Dromiciops gliroides* reveals adaptive regulation of protective functions during hibernation

Roberto F. Nespolo[1,2,3] | Juan Diego Gaitan-Espitia[4,5] | Julian F. Quintero-Galvis[1] | Fernanda V. Fernandez[6] | Andrea X. Silva[7] | Cristian Molina[7] | Kenneth B. Storey[8] | Francisco Bozinovic[2]

[1]Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

[2]Departamento de Ecología, Center of Applied Ecology and Sustainability (CAPES), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

[3]Millennium Institute for Integrative Biology (iBio), Santiago, Chile

[4]The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Hong Kong SAR, China

[5]CSIRO Oceans & Atmosphere, Hobart, Tasmania, Australia

[6]Instituto de Fisiología, Facultad de Medicina, Universidad Austral de Chile, Valdivia, Chile

[7]AUSTRALomics, Vicerrectoría de Investigación, Desarrollo y Creación Artística, Universidad Austral de Chile, Valdivia, Chile

[8]Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada

Correspondence
Roberto F. Nespolo, Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile.
Email: robertonespolorossi@gmail.com

Funding information
Fondo Basal CAPES 0002-2014; Fondecyt, Grant/Award Number: 1180917, 11140680; CSIRO-OCE Postdoctoral Fellowship; CONICYT PhD Fellowship, Grant/Award Number: 21160901; NSERC Canada, Grant/Award Number: 6793

Abstract

The small South American marsupial, *Dromiciops gliroides*, known as the missing link between the American and the Australian marsupials, is one of the few South American mammals known to hibernate. Expressing both daily torpor and seasonal hibernation, this species may provide crucial information about the mechanisms and the evolutionary origins of marsupial hibernation. Here, we compared torpid and active individuals, applying high-throughput sequencing technologies (RNA-seq) to profile gene expression in three *D. gliroides* tissues and determine whether hibernation induces tissue-specific differential gene expression. We found 566 transcripts that were significantly up-regulated during hibernation (369 in brain, 147 in liver and 50 in skeletal muscle) and 339 that were down-regulated (225 in brain, 79 in liver and 35 in muscle). The proteins encoded by these differentially expressed genes orchestrate multiple metabolic changes during hibernation, such as inhibition of angiogenesis, prevention of muscle disuse atrophy, fuel switch from carbohydrate to lipid metabolism, protection against reactive oxygen species and repair of damaged DNA. According to the global enrichment analysis, brain cells seem to differentially regulate a complex array of biological functions (e.g., cold sensitivity, circadian perception, stress response), whereas liver and muscle cells prioritize fuel switch and heat production for rewarming. Interestingly, transcripts of thioredoxin-interacting protein (*TXNIP*), a potent antioxidant, were significantly over-expressed during torpor in all three tissues. These results suggest that marsupial hibernation is a controlled process where selected metabolic pathways show adaptive modulation that can help to maintain homeostasis and enhance cytoprotection in the hypometabolic state.

KEYWORDS
adaptation, *Dromiciops*, functional genomics, hibernation, marsupials, RNA-seq

1 | INTRODUCTION

Endothermic animals (i.e., birds and mammals) produce metabolic heat in their bodies in a way that allows them to maintain a near-constant body temperature at values that are typically well above ambient temperature. This is an extravagant economy that requires these animals to maintain elevated energy budgets and spend a large part of their resources on basic maintenance. However, the benefits are large and allow endotherms to remain active in cold environments or travel long distances due to their high aerobic capacity, the only way to sustain long periods of activity (Koteja, 2004; Nespolo, Bacigalupe, Figueroa, Koteja, & Opazo, 2011). An adaptive strategy to ameliorate the high cost of endothermy is torpor, an energy-saving mechanism used by many small mammal and bird species, that involves a temporal interruption of endothermy that happens during cold periods (Boyer & Barnes, 1999; Ruf & Geiser, 2015). During torpor episodes, which can occur daily or seasonally (seasonal torpor is also known as hibernation, see reviews in Boyles et al., 2013; Ruf & Geiser, 2015), most normal biological functions are suppressed for periods ranging from overnight to several weeks. Animals show strong suppression of metabolic rate (often to values just 1%–10% of active levels; Ruf & Geiser, 2015), a decrease in body temperature to near ambient values, and experience reductions in most physiological processes (e.g., strongly reduced heartbeat and breathing rates). In these hypometabolic states, energy is re-allocated to some pathways that maintain organ function, whereas other processes are suppressed or interrupted. For instance, the brain, an organ that cannot be shut down without serious damage, receives about 10% of its normal perfusion during torpor but maintains neural activity, especially in the hypothalamus (Schwartz, Hampton, & Andrews, 2013). The liver, the metabolic centre of the body, is also important during torpor as this organ processes nutrients, detoxifies reactive oxygen species (ROS) and disposes toxic products, and produces multiple proteins and fuels for export to other tissues (Hadj-Moussa et al., 2016). Another important tissue, that shows reduced perfusion during torpor, is skeletal muscle. This tissue cannot be damaged as it is crucial for rewarming the body during arousal from hibernation (Hindle, Karimpour-Fard, Epperson, Hunter, & Martin, 2011).

The state of suspended animation characterizing torpor and hibernation (i.e., the "hibernation phenotype," Faherty, Villanueva-Canas, Klopfer, Alba, & Yoder, 2016) entails important risks for cells and tissues. A wealth of knowledge obtained from placental mammals has shown that torpor increases the risk of cardiac arrest and since blood perfusion to peripheral organs can be reduced, tissues can become hypoxic and ischaemic. This in turn increases the risk of oxidative damage especially resulting from a massive production of ROS during arousal (van Breukelen, Krumschnabel, & Podrabsky, 2010; Fons, Sender, Peters, & Jurgens, 1997; Rouble, Tessier, & Storey, 2014; Schwartz et al., 2013). In the brain, a reversible loss of synapses occurs during torpor, which reduces metabolic activity and helps to avoid the risk of neuronal death (Andrews, 2004; Schwartz et al., 2013). In skeletal muscle of the torpid animal, there are adaptive mechanisms minimizing muscular disuse atrophy which include differential regulation of genes related to protein biosynthesis and focal adhesion, which in turn helps to maintain muscle integrity and contractibility (Andres-Mateos et al., 2012; Fedorov et al., 2014; Hadj-Moussa et al., 2016). Several detailed studies, all performed in placental mammals (reviewed in Andrews, 2004; Carey, Andrews, & Martin, 2003; Morin & Storey, 2009; Villanueva-Canas, Faherty, Yoder, & Alba, 2014), have revealed that these changes involve transcriptional (gene expression), post-transcriptional (non-coding RNA), translational (protein synthesis) and post-translational (reversible protein modification) changes assisting these pro-survival measures. Here, we present a case of massive transcriptional changes, many of them with adaptive significance, occurring in a hibernating species of marsupial.

Marsupials shared a last common ancestor with placental mammals approximately 160 million years ago (Graves & Renfree, 2013; Renfree, 1981), and since then, they have diversified into a wide range of ecological niches, especially after the colonization of Australia in the late Cretaceous (Mitchell et al., 2014). Multiple small marsupial species exhibit torpor, which represents an evolutionary convergence with placental mammals (see Ruf & Geiser, 2015; Turner, Warnecke, Kortner, & Geiser, 2012). However, the underlying metabolic origins and patterns of marsupial hibernation are unclear. We know of three published studies describing some functional aspects of marsupial hibernation (Franco, Contreras, & Nespolo, 2013; Hadj-Moussa et al., 2016; Malan, 2010), which indicate some similarities with placental mammals (e.g., immunity suppression, mechanisms avoiding muscle atrophy, fuel switch to fat metabolism) but also some differences (e.g., a thermogenic role of the liver for rewarming and maintenance of the Akt metabolic pathway during torpor in the liver; Hadj-Moussa et al., 2016; Luu et al., 2018a; Villarin, Schaeffer, Markle, & Lindstedt, 2003). In this study, we used RNA-seq to analyse genomic-wide expression patterns of central and peripheral organs in the South American marsupial *Dromiciops gliroides*. This species is considered a "relict" mammal (sensu Habel, Assman, Schmidtt, & Avise, 2010) as it belongs to Microbiotheria, a formerly diverse group that diverged from Didelphimorphia (American marsupials) about 70 million years ago (MYA) and gave rise to Australidelphia, the large clade of Australian marsupials (Graves & Renfree, 2013; Mitchell et al., 2014). All Microbiotherids are extinct, excepting for *D. gliroides* (Palma & Spotorno, 1999).

According to Bozinovic, Ruiz, and Rosenmann (2004), *D. gliroides* is one of the few South American mammals that exhibit hibernation (=seasonal torpor, see also Geiser & Martin, 2013), but it also exhibits short torpor episodes during summer (i.e., daily torpor; Bozinovic et al., 2004; Nespolo, Verdugo, Cortes, & Bacigalupe, 2010). By the use of torpor, *D. gliroides* can save up to 60% of the energy that would otherwise be needed during the cold period. Previous work on *D. gliroides* suggested that torpor is associated with metabolic rate reductions of about 90% (Nespolo et al., 2010). During torpor in *D. gliroides*, a drastic redistribution of blood in the body induces

anaemia, leukopenia, muscle atrophy and inflammation (Franco et al., 2013).

The apparently random patterns of torpor that *D. gliroides* exhibit were formerly interpreted as acute, uncontrolled responses to cold (Nespolo et al., 2010). However, a number of recent discoveries have changed this view. For instance, *D. gliroides* seems to anticipate the cold season as a response to photoperiodic changes and thermal acclimation (Franco, Contreras, Place, Bozinovic, & Nespolo, 2017). In addition, several torpor-regulation mechanisms were described in this species, including differential expression microRNAs (Hadj-Moussa et al., 2016), implementation of the stress response through MAPK signalling (Luu et al., 2018b; Wijenayake et al., 2018a), reorganization of fuel use (Wijenayake et al., 2018b) and partial suppression of protein synthesis (Luu et al., 2018a). Here, we present a comprehensive transcriptomic analysis of torpid *D. gliroides*, providing the first explicit description of differentially regulated metabolic pathways of marsupial hibernation.

2 | METHODS

2.1 | Animal collection and laboratory treatment

Dromiciops gliroides is one of the four marsupial species of Chile; it is an omnivorous, nocturnal, opossum-like mammal with arboreal adaptations (i.e., opposable thumbs, prehensile tail and eyes in frontal plane; Hershkovitz, 1999). This species is strongly associated with the temperate rainforest, where temperatures fluctuate between 5 and 25°C (Franco et al., 2017). In this ecosystem, we captured thirteen adult *D. gliroides* (seven males; six females), particularly in the southern part of Valdivia, Chile (39°48′S, 73°14′W; 9 m.a.s.l.), during the austral summer (January–February) in 2014, using Tomahawk traps located in trees 1 m above ground, baited with bananas and yeast. Upon capture, individuals were immediately transported to the laboratory where they were housed in plastic cages of $45 \times 30 \times 20$ cm^3 with 2 cm of bedding. All individuals were maintained in a climate controlled chamber (PiTec Instruments, Chile) at 20 ± 1°C and with a 12-hr: 12-hr photoperiod for 2 weeks. Animals were fed a mix of mealworms, fruits and water ad libitum. After 2 weeks of acclimation, and after checking that each animal had increased body mass, individuals were randomly assigned to two groups: torpor (three males, four females) and active controls (three males, three females). Active animals were sampled from the above conditions. To induce torpor, and to avoid any injury, animals were subjected to a gradual decrease of ambient temperature (−1°C every 20 min) until 10°C was reached (photoperiod was maintained as initially). To minimize animal disturbance during the experimental trials, torpor incidence was verified by visual observation several times a day between 09:00–17:00. In this species, torpor can be easily identified: animals are not responsive when the cage is gently moved and breathing frequency is below three breaths per minute. After declaring torpor for a given individual, the animal was continuously monitored by visual inspection every 4 hr, during four consecutive days to ensure that torpor was sustained; and individuals were then euthanized. Euthanasia followed protocols approved by the Committee on the Ethics of Animal Experiments of the Universidad Austral de Chile. Tissue samples were excised in less than a minute and immediately frozen in liquid nitrogen. All animals capture, handling and maintenance procedures followed the guidelines of the American Society of Mammalogists (Gannon, Sikes, & Comm, 2007) and were authorized by the Chilean Agriculture and Livestock Bureau (SAG: Servicio Agrícola y Ganadero de Chile, permit No. 1054/2014 and 1118/2015).

2.2 | RNA extraction, cDNA library construction and sequencing

Total RNA was extracted from brain, liver and skeletal muscle from the hind leg (thigh) of each animal using the NucleoSpin RNA II Macherey Nagel kit (Bethlehem, PA, USA) and additional DNAase, following manufacturer's instructions. The quality of the obtained RNA was assessed by an Agilent 2100 Bioanalyzer. Only high-quality RNA with RNA integrity numbers (RINs over 7.5) was used (13 for brain, six for liver and four for skeletal muscle; 1:1 ratio of torpor: active organisms). RNA quantity was estimated using the Kit Quant-iTTM RiboGreen® RNA in a DQ300 Hoefer fluorometer. Individual cDNA libraries (N = 23) were labelled with sample-specific barcode adaptors, normalized and randomly built using the TrueSeq RNA Sample Preparation Kit v2 (Illumina; 0.5 μg of total RNA), following manufacturer's recommendations. These cDNA libraries were then pooled in equimolar ratios, with two or three randomly selected samples per pool, and were sequenced (2×150 bp PE) in 11 separated Illumina MiSeq runs at the AUSTRAL-*omics* Core Facility, Universidad Austral de Chile (www.australomics.cl). Randomization of library preparation and sequencing is described as a way to avoid confounding experimental factors with technical factors (Conesa et al., 2005). Sequences were demultiplexed based on their sample-specific barcode adaptors. Raw data from the sequencing runs were deposited at the Sequence Read Archive (SRA) repository of the National Center for Biotechnology Information (NCBI) under accession nos SRR6255590–SRR6255614 of the Bioproject PRJNA416414.

2.3 | Bioinformatics

Following sequencing, quality control (filtering and trimming) of the raw data was performed using the TRIMMOMATIC TOOL v.030 (Bolger, Lohse, & Usadel, 2014) and we removed every read with a phred quality score of 30 or less, which gives 99.9% in base accuracy. We used this phred score to be conservative and avoid multiple mappings, which could produce isoforms as artefacts of incorrect mismatches (see a debate in Williams, Baccarella, Parrish, & Kim, 2016). Still, some isoforms were produced which we interpret according to the involved biological function. The quality trimmed reads were assembled using Trinity 2.0.4 (Grabherr et al., 2011) with the standard Inchworm, Chrysalis and Butterfly pipeline and a minimum contig length of 200 nt (De Wit et al., 2012). These setting parameters

have been optimized for de novo assemblies of non-model species with Trinity (Grabherr et al., 2011). Duplicate sequences were then removed manually. The quality and completeness of the assembly were analysed using the software QUAST for assembly statistics (Gurevich, Saveliev, Vyahhi, & Tesler, 2013) and by mean of the Benchmarking Universal Single-Copy Orthologs (BUSCO v.3) approach (Simao, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015). For BUSCO, our analyses were based on a subset of 233 (Core Vertebrate Genes, CVG) and 4104 (Mammalia) orthologs, which in eukaryotes are widely conserved core genes that generally lack paralogs (Simao et al., 2015).

Processed high-quality reads were mapped to the assembled contigs using the BOWTIE (version 2.0) read aligner (Langmead & Salzberg, 2012). To improve isoform counts, we used the RNA-Seq by Expectation Maximization (RSEM, version 1.0) software (Li & Dewey, 2011) that assesses transcript abundance in the assembled transcriptome. Then, a sample-based clustering analysis (heatmap of Euclidean distances) was performed in order to identify the distribution of the samples according to the experimental conditions using the R function *dist* and the function *heatmap.2* from the GPLOTS package. Our de novo assembled transcriptome was blasted against the UniProt (Swiss-Prot and TrEMBL), KOBAS and NCBI RefSeq (nr) protein databases using the BLASTX algorithm with an e-value cut-off of $1e^{-5}$ (Altschul, Gish, Miller, Myers, & Lipman, 1990). With this procedure, the annotation was performed against a database containing several million proteins. Annotated unigenes (consensus, non-redundant sequences) were further searched for Gene Ontology (GO) terms using BLAST2GO software (www.blast2go.com; Conesa et al., 2005) according to the main categories of Gene Ontology (GO; molecular functions, biological processes and cellular components; Ashburner et al., 2000). Complementary annotations were done with the INTERPROSCAN v.5 software (Jones et al., 2014), which provides functional analysis of proteins by classifying them into families and predicting domains and important sites. The annotation results were further fine-tuned with the Annex and GO slim functions of the BLAST2GO software in order to improve and summarize the functional information of the transcriptome data set. Additionally, proteins were finally annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and its automated assignment server (KAAS; Moriya, Itoh, Okuda, Yoshizawa, & Kanehisa, 2007).

2.4 | Differential gene expression analysis

Differentially expressed genes (DEGs) were identified using the R/BIOCONDUCTOR package DESEQ2 v.1.10 (Love, Huber, & Anders, 2014) with raw read counts. The estimated counts were normalized against the size of the transcriptome and the total number of readings that were mapped per sample, using the regularized logarithm (rlog) method in DESEQ2 and expressed in a log2 scale. Basically, DESEQ2 normalizes the counts by dividing each column of the count table (samples) by the size factor of this column. The size factor is then calculated by dividing the samples by the geometric means of the genes, which brings the count values to a common scale suitable for comparison (Love et al., 2014). *p*-Values for differential expression were calculated using a negative binomial test for differences between the base means of the control and torpor conditions. The *p*-values were adjusted for multiple test correction using Ward's method with the Benjamini–Hochberg procedure (Ferreira & Zwinderman, 2006). Significant DEGs were defined as those genes with an adjusted *p*-value (false discovery rate, FDR) ≤ 0.05 and $\log_2$ (fold change) ≥ 1. Differentially expressed genes across samples were visualized using standard volcano plots, where $\log_2$ fold change was plotted against $\log_{10}$ (FDR adjusted *p*-value). Furthermore, heatmaps were produced to visualize gene expression across samples and tissues using *z*-scores (based on normalized counts) and plotted with the HEATMAPPER software (Babicki et al., 2016).

Enrichment of GO and KEGG pathways in genes up- and down-regulated during torpor was analysed using BLAST2GO (Fisher's exact test) and the GOSEQ R package (Young, Wakefield, Smyth, & Oshlack, 2010), with a threshold false discovery rate of 0.001. The reference used was the whole transcripts with GO slim annotation. Chord diagrams to visualize enriched pathways were drawn using Circos (Krzywinski et al., 2009).

3 | RESULTS

In this study, a total of 414 million of reads were generated from 23 libraries derived from brain (13), liver (6) and skeletal muscle (4) of active and hibernating *D. gliroides* (mean = 8.6 million of reads per sample; see Supporting Information Table S1). After a stringent filtering process, ~94% high-quality, adapter-free and non-redundant reads were retained for further downstream analyses. Our de novo assembly generated 507,815 contiguous sequences (putative transcripts, contigs) with a mean sequence length of 718 bp, an N50 of 1,387 bp and an L50 of 60,430. The longest sequence contains 68,683 bp, and 16% of the sequences were over 1k bp. The assessment of transcriptome completeness using the Benchmarking Universal Single-Copy Orthologs (BUSCO) approach identified a high representation of Core Vertebrate Genes (CVG), with 94.4% marked as complete and 98.1% as complete + partial. Only 1.29% of the CVG were missing. Similarly, our BUSCO analysis revealed 3,577 (87%) complete and 3,929 (95.74%) complete + partial Mammalian Core Genes (MCG). From this reference gene set, 175 (4.26%) MCG were missing in our de novo assembly. In terms of the functional association of the putative transcripts in the de novo assembled transcriptome of *D. gliroides*, our analysis produced 31,438 contigs that were blasted to known proteins in the public databases NCBI (nr), KOBAS and UniProt (Swiss-Prot and TrEMBL) was linked to GO classifications (average 4.55 GOs per contig). Hypothetical or predicted proteins in these databases were excluded by discarding matches associated with "hypothetical," "predicted," "unknown" and "putative" categories. Most of the annotated contigs (93%) hit against the koala (*Phascolarctos cinereus*), the grey short-tailed opossum (*Monodelphis domestica*) and the Tasmanian devil (*Sarcophilus harrisii*) genomes, in this order.

Our transcriptomic survey of hibernating *D. gliroides* identified 73,125 mRNA transcripts in the brain, of which 594 exhibited differential regulation during torpor; 225 of them were down-regulated and 369 up-regulated (Figure 1a). Some of the very highly differentially expressed genes are named on the figure. In the liver, we identified 36,865 transcripts with 226 showing differential regulation during torpor: 79 down-regulated and 147 up-regulated (Figure 1b). In skeletal muscle, these numbers were 13,038 total transcripts with 85 differentially regulated during torpor: 35 down-regulated and 50 up-regulated (Figure 1c). We found 317 transcripts that were exclusively up-regulated in the brain, 131 transcripts that were exclusively up-regulated in the liver, and 44 transcripts exclusively up-regulated in muscle (Figure 1d; upper panel). Oppositely, 191 transcripts were exclusively down-regulated in the brain, 73 in the liver and 46 in muscle (Figure 1d; lower panel). A few transcripts were up-regulated or down-regulated in common among two or all three of the organs; these are named in Figure 1d; and more details about their functions are given in Supporting Information Tables S2–S7. For example, *SETDB1*, *SCL25A18 and ACADVL* were up-regulated in both brain and liver whereas *EIF2AK1* was up-regulated in both brain and muscle. Only one transcript, encoding thioredoxin-interacting protein (*TXNIP*; Figure 1), was up-regulated in common in all three tissues and also fell within the top 10 up-regulated genes in each of these organs (see Supporting Information Tables S1–S7). This gene is described as encoding potent antioxidant protein associated with a number of human diseases (see Discussion).

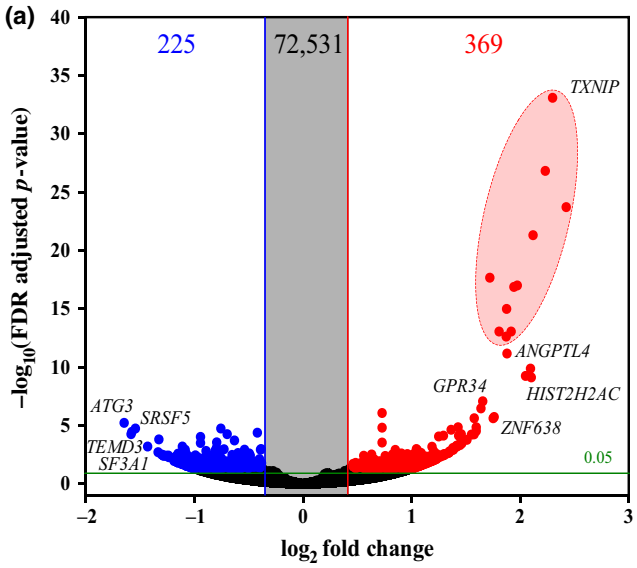
Functions such as protection against reactive oxygen species (gene: *TXNIP*; over-expressed in all three organs of hibernators: Figures 1a–c and 2a), inhibition of transcription (genes: *HIST2H2A*; *SRSF5*; Figures 1a and 2), fuel switch to fat metabolism (genes: *ZNF638*, *ATG3*; Figures 1d,f and 2) and inhibition of angiogenesis (*ANGPTL4*; Figure 1c), appeared as the most important changes in the brain (Figure 1; Supporting Information Tables S2 and S3). In the liver, the greatest changes in gene expression characterizing torpor seemed to be associated with the fuel switch from carbohydrate to lipid catabolism, since four genes involved in promoting fat catabolism enzymes were among the top five differentially expressed ones (*PDP2*, *CYB5R3*, over-expressed; *NR1H4*, *ND4*, under-expressed, Supporting Information Tables S4 and S5). In muscle, a similar interpretation indicated that mechanisms for avoiding muscle atrophy (over-expressed genes: *PVALB*, *EIF3D*, *GADPH*, Supporting Information Figure S4; Supporting Information Tables S4 and S5) may be the most important functions being exacerbated during torpor.

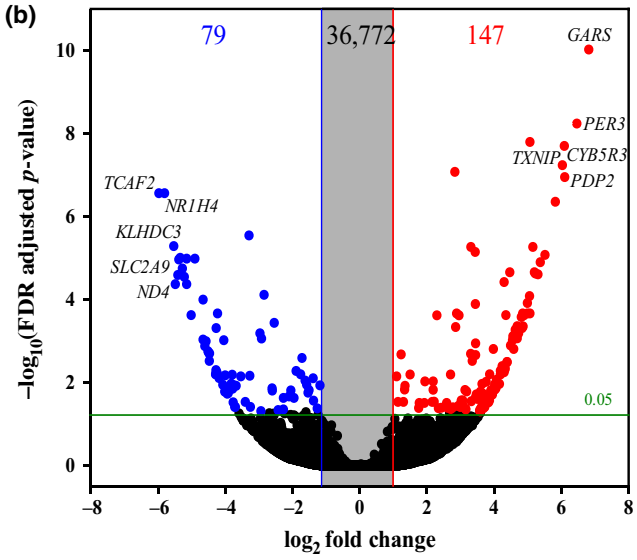
A functional enrichment analysis based on the gene ontology database (GO) suggested that several metabolic pathways were enriched (both under-expressed and over-expressed) in the brain during torpor, compared with the other two organs, that only showed overexpression of a few biological functions (Figure 2a). This is also appreciated in the expression profiles of each organ (i.e., the "heatmaps," see Supporting Information Figure S4). The analysis arising from the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed a myriad of functions that were differentially regulated in the brain, such as cold sensitivity, circadian perception, mRNA surveillance and stress response (Figure 2b). The liver and muscle profile, by contrast, indicated that the most important modified functions were orientated to the maintenance of organ function (e.g., biosynthesis of amino acids) and to fuel switch to lipid metabolism (e.g., fatty acid degradation, metabolic pathways; Figure 2c,d).

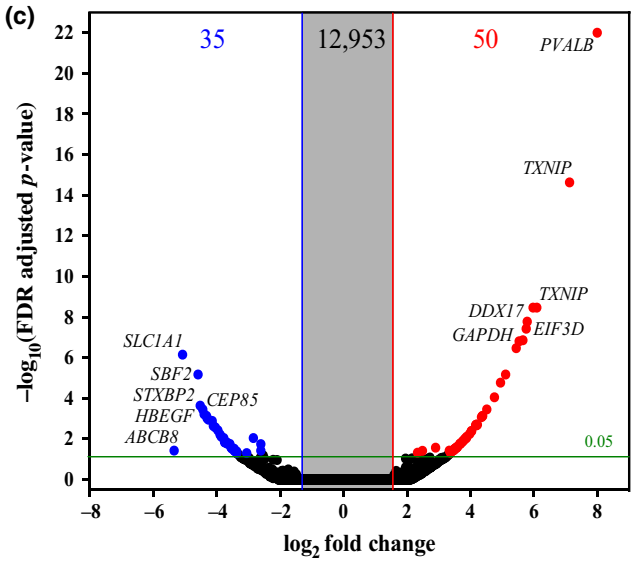
4 | DISCUSSION

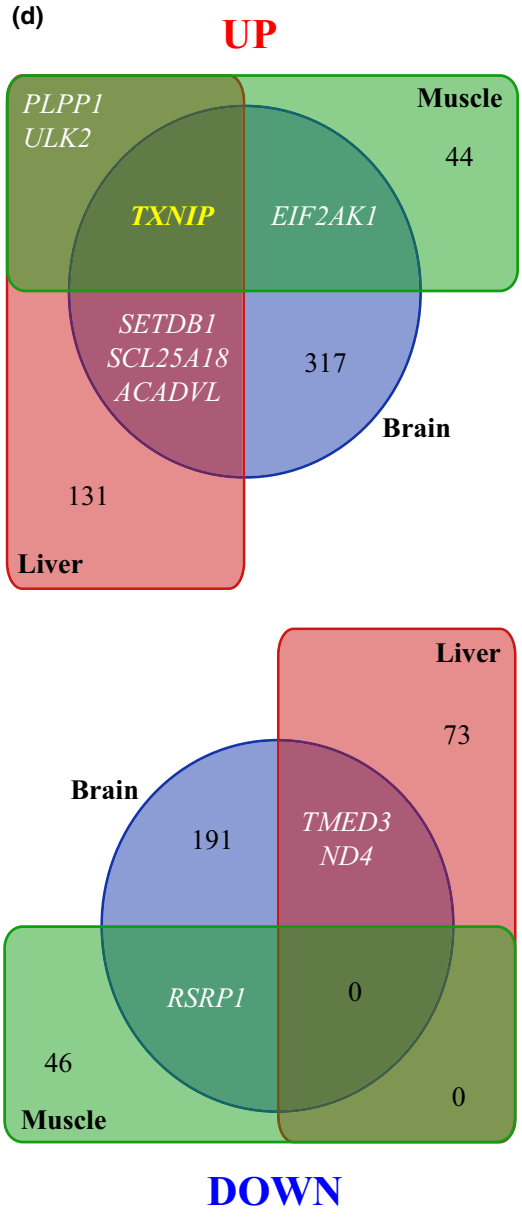
Today, comparative physiologists have a broad repertoire of technological tools that can be used to identify functional changes associated with a given physiological condition; from simple (and often inexpensive) measures of whole-animal metabolic fluxes (e.g., respirometry, blood biochemistry and haematology, tissue-specific enzymes and metabolites; see recent examples in Franco et al., 2013; Il'ina et al., 2017; Rouble & Storey, 2015) to the powerful characterization of exacerbated/enriched metabolic pathways that high-throughput sequencing methods provide. To the best of our knowledge, this is the first RNA-seq analysis of hibernation in a marsupial, which provided a wealth of detailed information. In order to avoid being "lost in the map" (sensu Travisano & Shaw, 2013), we focus on some particularly important metabolic functions with relevance for torpor, provided by our de novo assembly. This procedure showed high completeness as evidenced for the percentage of coverage of Core Vertebrate Genes (CVG) and Mammalian Core Genes (MCG). The overall statistics of our assembly (N50, L50, contig length, number of contigs >1k) were similar to the results documented in de novo assembled transcriptomes of other mammals, such as the beaver (*Castor fiber L.*; testis; Bogacka et al., 2017), and the Nile grass rat (*Arvicanthis ansorgei*; retina; Liu et al., 2017). However, we had higher values compared with marsupials such as the long-nosed bandicoot (*Perameles nasuta*; heart, liver, spleen and kidney; Morris et al., 2018) and the Virginia opossum *Didelphis virginiana*; kidney; Eshbach et al., 2017).

FIGURE 1 (a–c) Volcano plots showing differentially regulated genes at the $p = 0.05$ level (green, horizontal line) in three tissues of torpid *Dromiciops gliroides* as compared with active animals. Significantly down-regulated genes are indicated as negative fold change (blue), and up-regulated genes are indicated as positive values (red). The grey zone indicates the number of transcripts that do not show significant differential expression. (a) brain; (b) liver; (c) skeletal muscle. (d) commonly up-regulated genes among organs (upper panel) and commonly down-regulated genes (bottom panel). The numbers represent the numbers of transcripts that were differentially regulated exclusively for each organ (e.g., 44 transcripts were exclusively and significantly up-regulated in muscle). Most differentially regulated genes are written in yellow and white font on the diagrams. Descriptions of the top 10 significantly regulated genes are provided in Supporting Information Tables S2–S7). Several isoforms of the *TXNIP* gene were found among the up-regulated genes in brain, which are denoted by the red ellipse (a)


(a)
225
72,531
369
TXNIP
ANGPTL4
GPR34
HIST2H2AC
ZNF638
ATG3
SRSF5
TEMD3
SF3A1
0.05
−log10(FDR adjusted p-value)
log2 fold change


(b)
79
36,772
147
GARS
PER3
TXNIP
CYB5R3
PDP2
TCAF2
NR1H4
KLHDC3
SLC2A9
ND4
0.05
−log10(FDR adjusted p-value)
log2 fold change


(c)
35
12,953
50
PVALB
TXNIP
TXNIP
DDX17
EIF3D
GAPDH
SLC1A1
SBF2
STXBP2
CEP85
HBEGF
ABCB8
0.05
−log10(FDR adjusted p-value)
log2 fold change


(d)
UP
PLPP1
ULK2
Muscle
44
TXNIP
EIF2AK1
SETDB1
SCL25A18
ACADVL
317
Brain
131
Liver
Liver
73
Brain
191
TMED3
ND4
RSRP1
0
46
0
Muscle
DOWN

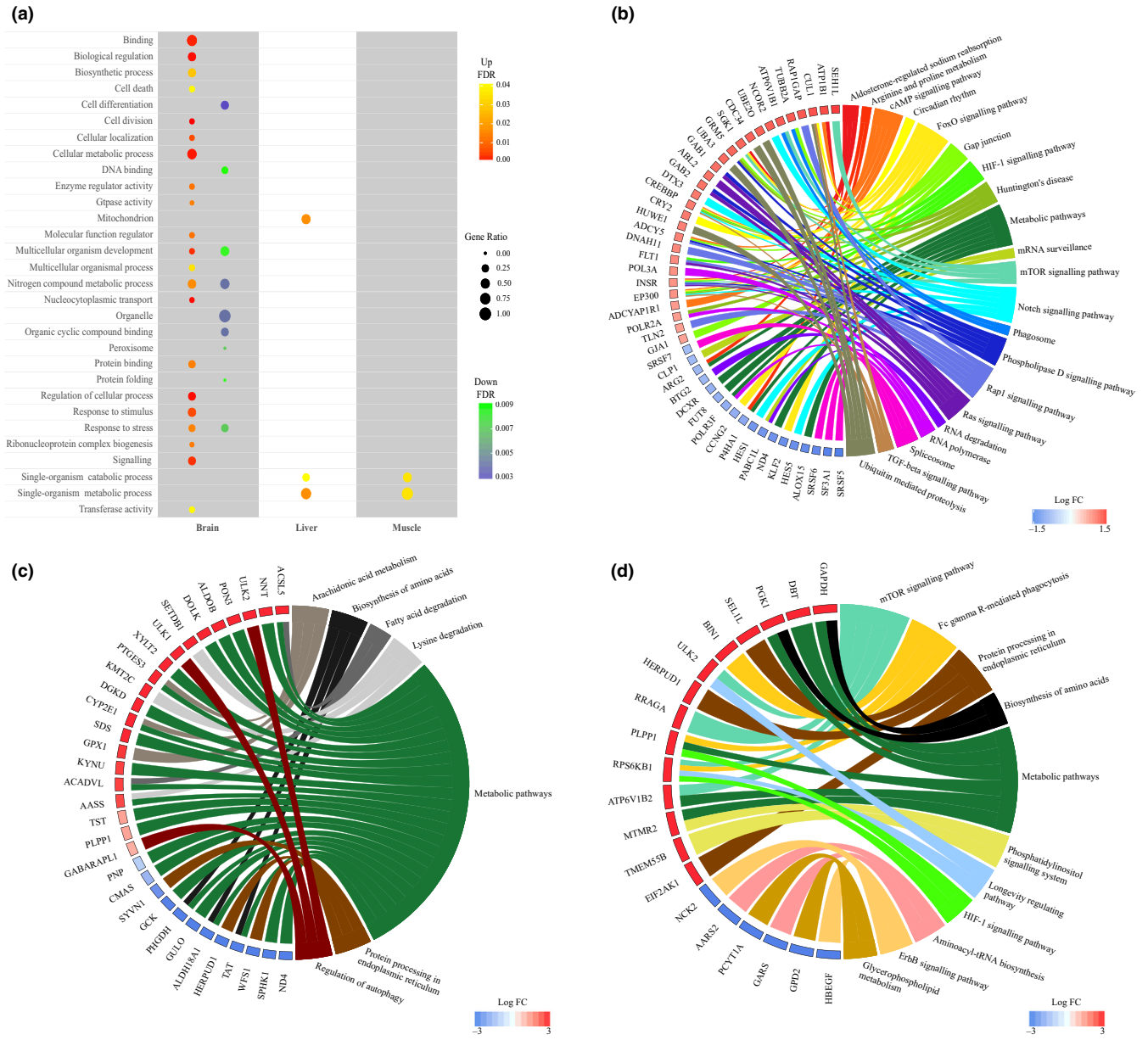


FIGURE 2 Functional enrichment analysis of genes that appeared over-represented during torpor using the gene ontology database (a). The size of the circles represents the number of differentially expressed genes over the total number of genes, associated with a given GO term, whereas the colour indicates the level of significance. Also, a functional enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG database) is shown for brain (b), liver (c) and muscle (d). In this analysis, genes are indicated at the left side of each pie graph, with the respective level of expression (Log2 FC) indicated in colour in the small squares, and metabolic pathways are indicated in the right side of the graph, connected to the group of genes that are associated with the pathway by lines

4.1 | Thioredoxin-interacting protein and oxidative damage

The most notable finding of our analysis was the overexpression in all three organs of *TXNIP*, the gene encoding thioredoxin-interacting protein. The TXNIP was first identified as an endogenous negative regulator of thioredoxin, a ubiquitous redox protein in cells that is particularly involved in the reduction of oxidized cysteine residues and cleavage of disulphide bonds (Nishiyama et al., 1999). TXNIP has been linked, not just with an antioxidant/redox role (e.g., to minimize ischaemia–reperfusion damage), but with the broader regulation of mitochondrial function to help suppress oxidative metabolism when oxygen is limiting, and shift metabolism to anaerobic glucose catabolism by mediating inhibition of pyruvate dehydrogenase (Chong et al., 2014; Spindel, World, & Berk, 2012; Yoshioka & Lee, 2014). Several diseases are associated with disruptions of the thioredoxin system, such as cataract formation, ischaemic heart diseases, several cancers, diabetes complication and hypertension (Maulik & Das, 2008). TXNIP is also involved in inhibiting unnecessary glucose influx into cells while also promoting fatty acid oxidation (Hand et al., 2013); both of these

are central features of a hibernating phenotype. Indeed, recent research has shown that the *TXNIP* gene was over-expressed in brain (hypothalamus), liver and white and brown adipose during induced-torpor experiments in mice as well as in natural torpor in Siberian hamsters (*Phodopus sungorus*; DeBalsi et al., 2014; Hand et al., 2013; Jastroch et al., 2016). Our current identification of a multi-organ strong up-regulation of *TXNIP* in *D. gliroides* (including multiple gene variants in brain) adds further support for the proposal that TXNIP has a central role in the metabolic control of torpor.

4.2 | Metabolic switch

In the brain, ANGPTL4 secretion (which we found strongly up-regulated) is of central importance in regulating the switch to a lipid-based fuel economy during torpor, facilitating lipid release from adipose and uptake by other tissues. Indeed, recent studies have reported significant up-regulation of *ANGPTL4* transcripts in ground squirrel heart during torpor and interbout arousal stages of hibernation as compared with pre- or post-hibernation months (Vermillion, Anderson, Hampton, & Andrews, 2015) as well as during torpor in a ground squirrel bone marrow transcriptome when compared with summer animals (intermediate transcript levels were seen during interbout arousal; Cooper et al., 2016). In the same vein, a powerful indicator of the suppression of carbohydrate fuel use within the brain during torpor is pyruvate dehydrogenase kinase 4 (*PDK4*), whose transcripts were strongly elevated in the brain. Phosphorylation of pyruvate dehydrogenase (PDH) at S232, S293 or S300 by any of four PDK isozymes inhibits its activity (Harris, Bowker-Kinley, Huang, & Wu, 2002) and is crucial for blocking the oxidation of pyruvate as a substrate, especially when carbohydrate reserves must be conserved. Indeed, strong suppression of PDH activity during hibernation has been widely reported in multiple tissues of eutherian hibernators (summarized in Wijenayake, Tessier, & Storey, 2017). Strong increases in PDH phosphorylation at 1, 2 or all 3 serine sites were also reported for six tissues (including brain, liver and skeletal muscle) of *D. gliroides* (Wijenayake et al., 2017) and the up-regulation of *PDK4* in brain (predictably elevating PDK4 protein) would support PDH inhibition and presumably help to direct brain to make greater use of ketones as substrates during hibernation.

4.3 | Marsupial nonshivering thermogenesis

Uniquely in marsupials, liver appears to be the main site of nonshivering thermogenesis since brown adipose tissue is not present (Jastroch, Wuertz, Kloas, & Klingenspor, 2005; Rose, West, Ye, McCormack, & Colquhoun, 1999) and, hence, modulation of multiple controls on lipid metabolism is probably needed to regulate this novel liver function (Hadj-Moussa et al., 2016). Among down-regulated genes we found in liver, three deserve particular mention for their potential roles in the hibernating marsupial: *ND4*, *NR1H4* and *TCAF2* (see Supporting Information Table S5). Transcript levels of the mitochondria-encoded NADH dehydrogenase subunit 4 (*ND4*) gene were strongly reduced in *D. gliroides* liver during hibernation. By contrast, strong increases in ND4 expression were reported in brown adipose tissue of the bat, *Myotis lucifugus* during hibernation (Eddy, Morin, & Storey, 2006) and *ND2* transcripts (also mitochondria-encoded) were elevated during hibernation in heart and skeletal muscle of 13-lined ground squirrels, *Spermophilus tridecemlineatus* (Fahlman, Storey, & Storey, 2000). Compared with *D. gliroides*, this suggests that there may be either tissue-specific (liver vs. muscle/BAT) or marsupial versus eutherian differences in the reorganization of mitochondrial oxidative metabolism in the torpid state. On the other hand, *NR1H4* encodes the NR1H4 protein (nuclear receptor subfamily 1, group H, member 4) that is also known as the bile acid receptor (BAR) or the farnesoid X receptor (FXR). This receptor is a master regulator of hepatic triglyceride, cholesterol and bile acid metabolism. Active FXR exerts controls that suppress de novo lipogenesis and promote FFA oxidation. FXR gene expression was also reduced in liver of hibernating ground squirrels compared with summer animals (Nelson, Otis, & Carey, 2009) and also occurs in non-alcoholic fatty liver disease in humans. FXR-deficient mice not only exhibited marked hepatosteatosis (fatty liver) and hypertriglyceridaemia (Jiao, Lu, & Li, 2015; Wollam & Antebi, 2011) but showed an accelerated fasting-induced entry into torpor and markedly greater cold intolerance as compared with controls (Cariou et al., 2007). Hence, the strong suppression of *NR1H4* transcript levels (implying suppressed FXR protein levels) in liver of hibernating *D. gliroides* suggests a role for this receptor in the management and/or restructuring of liver lipid metabolism during hibernation when fatty acid oxidation is the primary mode of ATP production. This, together with previous results in *D. gliroides* and also in *Monodelphis domestica* (Hadj-Moussa et al., 2016; Villarin et al., 2003), provides an intriguing role between FXR (BAR) function, lipid metabolism and NST in the liver metabolism of hibernating marsupials.

4.4 | KEGG integrated analysis

The analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG, see Figure 2b) showed, in torpid animals, overexpression of multiple genes contributing to the mTOR signalling pathway (genes *SEH1L*, *SGK1*), circadian rhythm pathways (genes *CUL1*, *CRY2*), notch signalling pathway (genes *NCOR2*, *DTX3*, *EP300*), and ubiquitin-mediated proteolysis (genes *CUL1*, *UBE20*, *CDC34*, *UBA3*, *HUWE1*). Seh1 (known as SEH1L in mammals) is a subunit of the GATOR2 complex that is an essential activator of mTORC1 kinase. Seh1 is also a subunit of the Nup107 complex (the nucleoporin Y-complex) that plays a major role in formation of the nuclear pore complex in interphase and associates with kinetochores in mitosis (Platani, Samejima, Samejima, Kanemaki, & Earnshaw, 2018). SGK1, on the other hand, is one of many downstream targets of the mTOR C2 kinase, representing one arm of the mTORC2 signalling pathway (Garcia-Martinez & Alessi, 2008). Cry2 is one of the main circadian rhythm proteins, and it is known that this protein is up-regulated during hibernation in hamsters and ground squirrels (Crawford et al., 2007).

The high level of transcriptional activity detected in the brain contrasts with the few enriched pathways of liver and muscle

(Figure 2c,d). This, however, could be a consequence of the low sample size we had for those two organs (especially for muscle), which makes our conclusions regarding these organs, preliminary. Both for liver and muscle we found a strong differential regulation (up- and down-regulation) of metabolic pathways sensu *lato*, which is probably due to the physiological switch from carbohydrate to lipid-based metabolism also described in other hibernators (Boyer & Barnes, 1999; Storey & Storey, 2010; Villanueva-Canas et al., 2014), and in *D. gliroides* (Wijenayake et al., 2018b). This is confirmed here, as we found strong overexpression of pathways related to fatty acid degradation (genes *ACSL5*, *ACADVL*) and regulation of autophagy (genes *ULK1*, *ULK2*, *GABARAPL1*) in the liver (see Figure 2c). Hibernators all increase their content of unsaturated FAs so that lipid depots can remain fluid at low Tb (Contreras, Franco, Place, & Nespolo, 2014; Rose, Epperson, Carey, & Martin, 2011). Our findings support this view, since differential up-regulation of ACSL5 (the protein acyl-CoA synthetase long-chain 5) is used both in fatty acid synthesis and beta-oxidation. By contrast, in muscle we found overexpression of the longevity-regulating pathway, which indicates that differentially expressed genes in the muscle are directed towards the maintenance of organ function, which in marsupials (in addition to the liver, as discussed before) is crucial for rewarming (Hadj-Moussa et al., 2016; Opazo, Nespolo, & Bozinovic, 1999).

5 | SUMMARY AND CONCLUSIONS

In this paper, we have shown that the hibernating marsupial *D. gliroides* express adaptive physiological mechanisms to deal with the consequences of hypometabolism and cold during torpor. These mechanisms are tissue-specific and involve: (a) protection against reactive oxygen species, ROS (i.e., oxidative damage) by overexpressing the *TXNIP* gene among others, (b) metabolic switch from carbohydrate to fat-based metabolism in liver and muscle, (c) nonshivering thermogenesis in the liver, (d) transcriptional suppression of nonessential functions, (e) overexpression of proteins controlling circadian rhythm in the brain, and (f) overexpression of longevity-regulated pathways that maintain organ function in muscle. In terms of survival and fitness, these physiological changes generate the net consequence of making this metabolic depression, reversible and safe. Several of these mechanisms are conserved, previously described in placental mammals, but also described in *D. gliroides*. Some of them are apparently unique to marsupials (e.g., role of liver in rewarming), but still only described in a few species. Given that Microbiotherids are considered the ancestors of Australian marsupials (Mitchell et al., 2014), further studies in other marsupial species would be crucial to determine the generality of our findings.

ACKNOWLEDGEMENTS

This study was funded by Fondo Basal CAPES 0002-2014 to Francisco Bozinovic and Fondecyt grant no. 1180917 to Roberto Nespolo. Juan Diego Gaitan acknowledges a CSIRO-OCE Postdoctoral Fellowship. Julian F. Quintero-Galvis acknowledges a CONICYT PhD fellowship no. 21160901. Andrea Silva acknowledges a Fondecyt grant no. 11140680. Kenneth B. Storey acknowledges NSERC Canada grant no. 6793.

DATA ACCESSIBILITY

The data presented in this paper are now publicly available in ZENODO (https://doi.org/10.5281/zenodo.1409041) whereas the raw data from the sequencing runs were deposited at the Sequence Read Archive (SRA) repository of the National Center for Biotechnology Information (NCBI) under accession nos SRR6255590–SRR6255614 of the Bioproject PRJNA416414.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

R.F.N. designed the study, contributed to experimental execution and wrote the manuscript. J.D.G.-E. contributed to experimental design and execution, performed the final bioinformatic analysis, constructed the two main figures of the paper and edited versions of the manuscript. J.F.Q.-G. collaborated with the experiments, contributed to data analysis and edited versions of the manuscript. F.V.F. edited versions of the manuscript. A.X.S. contributed to experimental execution and bioinformatic analysis. C.M. performed the initial bioinformatic analysis and contributed to editing the methods section. K.B.S. contributed to editing versions of the manuscript and the discussion regarding gene function in the hibernating phenotype. F.B. funded the study and contributed to experimental design and editing of the manuscript.

ORCID

Roberto F. Nespolo http://orcid.org/0000-0003-0825-9618
Juan Diego Gaitan-Espitia http://orcid.org/0000-0001-8781-5736
Julian F. Quintero-Galvis http://orcid.org/0000-0001-9337-0606
Andrea X. Silva http://orcid.org/0000-0002-4346-5524
Kenneth B. Storey http://orcid.org/0000-0002-7363-1853
Francisco Bozinovic http://orcid.org/0000-0003-3870-9624

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1006/jmbi.1990.9999

Andres-Mateos, E., Mejias, R., Soleimani, A., Lin, B. M., Burks, T. N., Marx, R., … Cohn, R. D. (2012). Impaired skeletal muscle regeneration in the absence of fibrosis during hibernation in 13-lined ground squirrels. *PLoS One*, *7*(11), e48884. https://doi.org/10.1371/journal.pone.0048884

Andrews, M. T. (2004). Genes controlling the metabolic switch in hibernating mammals. *Biochemical Society Transactions*, *32*, 1021–1024. https://doi.org/10.1042/BST0321021

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., … Gene Ontology Consortium (2000). Gene Ontology: Tool for the unification of biology. *Nature Genetics*, *25*(1), 25–29. https://doi.org/10.1038/75556

Babicki, S., Arndt, D., Marcu, A., Liang, Y. J., Grant, J. R., Maciejewski, A., & Wishart, D. S. (2016). Heatmapper: Web-enabled heat mapping for all. *Nucleic Acids Research*, *44*(W1), W147–W153. https://doi.org/10.1093/nar/gkw419

Bogacka, I., Paukszto, T., Jastrzebski, J. P., Czerwinska, J., Chojnowska, K., Kaminska, B., … Kaminski, T. (2017). Seasonal differences in the testicular transcriptome profile of free-living European beavers (*Castor fiber* L.) determined by the RNA-Seq method. *PLoS One*, *12*(7), 20. https://doi.org/10.1371/journal.pone.0180323

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Boyer, B. B., & Barnes, B. M. (1999). Molecular and metabolic aspects of mammalian hibernation. *BioScience*, *49*, 713–724. https://doi.org/10.2307/1313595

Boyles, J. G., Thompson, A. B., McKechnie, A. E., Malan, E., Humphries, M. M., & Careau, V. (2013). A global heterothermic continuum in mammals. *Global Ecology and Biogeography*, *22*(9), 1029–1039. https://doi.org/10.1111/geb.12077

Bozinovic, F., Ruiz, G., & Rosenmann, M. (2004). Energetics and torpor of a South American "living fossil", the Microbiotheriid *Dromiciops gliroides*. *Journal of Comparative Physiology B*, *174*, 293–297. https://doi.org/10.1007/s00360-004-0414-8

van Breukelen, F., Krumschnabel, G., & Podrabsky, J. E. (2010). Vertebrate cell death in energy-limited conditions and how to avoid it: What we might learn from mammalian hibernators and other stress-tolerant vertebrates. *Apoptosis*, *15*(3), 386–399. https://doi.org/10.1007/s10495-010-0467-y

Carey, H. V., Andrews, M. T., & Martin, S. L. (2003). Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiological Reviews*, *83*(4), 1153–1181. https://doi.org/10.1152/physrev.00008.2003

Cariou, B., Bouchaert, E., Abdelkarim, M., Dumont, J., Caron, S., Fruchart, J. C., … Staels, B. (2007). FXR-deficiency confers increased susceptibility to torpor. *FEBS Letters*, *581*(27), 5191–5198. https://doi.org/10.1016/j.febslet.2007.09.064

Chong, C. R., Chan, W. P. A., Nguyen, T. H., Liu, S. F., Procter, N. E. K., Ngo, D. T., … Horowitz, J. D. (2014). Thioredoxin-interacting protein: Pathophysiology and emerging pharmacotherapeutics in cardiovascular disease and diabetes. *Cardiovascular Drugs and Therapy*, *28*(4), 347–360. https://doi.org/10.1007/s10557-014-6538-5

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., & Robles, M. (2005). Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*(18), 3674–3676. https://doi.org/10.1093/bioinformatics/bti610

Contreras, C., Franco, M., Place, N. J., & Nespolo, R. F. (2014). The effects of poly-unsaturated fatty acids on the physiology of hibernation in a South American marsupial, *Dromiciops gliroides*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, *177*, 62–69. https://doi.org/10.1016/j.cbpa.2014.07.004

Cooper, S. T., Sell, S. S., Fahrenkrog, M., Wilkinson, K., Howard, D. R., Bergen, H., … Hampton, M. (2016). Effects of hibernation on bone marrow transcriptome in thirteen-lined ground squirrels. *Physiological Genomics*, *48*(7), 513–525. https://doi.org/10.1152/physiolgenomics.00120.2015

Crawford, F. I. J., Hodgkinson, C. L., Ivanova, E., Logunova, L. B., Evans, G. J., Steinlechner, S., & Loudon, A. S. I. (2007). Influence of torpor on cardiac expression of genes involved in the circadian clock and protein turnover in the Siberian hamster (*Phodopus sungorus*). *Physiological Genomics*, *31*(3), 521–530. https://doi.org/10.1152/physiolgenomics.00131.2007

De Wit, P., Pespeni, M. H., Ladner, J. T., Barshis, D. J., Seneca, F., Jaris, H., … Palumbi, S. R. (2012). The simple fool's guide to population genomics via RNA-Seq: An introduction to high-throughput sequencing data analysis. *Molecular Ecology Resources*, *12*(6), 1058–1067. https://doi.org/10.1111/1755-0998.12003

DeBalsi, K. L., Wong, K. E., Koves, T. R., Slentz, D. H., Seiler, S. E., Wittmann, A. H., … Lark, D. S. (2014). Targeted metabolomics connects thioredoxin-interacting protein (TXNIP) to mitochondrial fuel selection and regulation of specific oxidoreductase enzymes in skeletal muscle. *Journal of Biological Chemistry*, *289*(12), 8106–8120. https://doi.org/10.1074/jbc.M113.511535

Eddy, S. F., Morin, P. J. R., & Storey, K. B. (2006). Differential expression of selected mitochondrial genes in hibernating little brown bats, *Myotis lucifugus*. *Journal of Experimental Zoology Part A-Comparative Experimental Biology*, *305A*(8), 620–630. https://doi.org/10.1002/jez.a.294

Eshbach, M. L., Sethi, R., Avula, R., Lamb, J., Hollingshead, D. J., Finegold, D. N., … Weisz, O. A. (2017). The transcriptome of the *Didelphis virginiana* opossum kidney OK proximal tubule cell line. *American Journal of Physiology-Renal Physiology*, *313*(3), F585–F595. https://doi.org/10.1152/ajprenal.00228.2017

Faherty, S. L., Villanueva-Canas, J. L., Klopfer, P. H., Alba, M. M., & Yoder, A. D. (2016). Gene expression profiling in the hibernating primate, *Cheirogaleus medius*. *Genome Biology and Evolution*, *8*(8), 2413–2426. https://doi.org/10.1093/gbe/evw163

Fahlman, A., Storey, J. M., & Storey, K. B. (2000). Gene up-regulation in heart during mammalian hibernation. *Cryobiology*, *40*(4), 332–342. https://doi.org/10.1006/cryo.2000.2254

Fedorov, V. B., Goropashnaya, A. V., Stewart, N. C., Toien, O., Chang, C., Wang, H. F., … Barnes, B. M. (2014). Comparative functional genomics of adaptation to muscular disuse in hibernating mammals. *Molecular Ecology*, *23*(22), 5524–5537. https://doi.org/10.1111/mec.12963

Ferreira, J. A., & Zwinderman, A. H. (2006). On the Benjamini-Hochberg method. *Annals of Statistics*, *34*(4), 1827–1849. https://doi.org/10.1214/009053606000000425

Fons, R., Sender, S., Peters, T., & Jurgens, K. D. (1997). Rates of rewarming, heart and respiratory rates and their significance for oxygen transport during arousal from torpor in the smallest mammal, the etruscan shrew *Suncus etruscus*. *Journal of Experimental Biology*, *200*, 1451–1458.

Franco, M., Contreras, C., & Nespolo, R. F. (2013). Profound changes in blood parameters during torpor in a South American marsupial. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, *166*(2), 338–342. https://doi.org/10.1016/j.cbpa.2013.07.010

Franco, M., Contreras, C., Place, N. J., Bozinovic, F., & Nespolo, R. F. (2017). Leptin levels, seasonality and thermal acclimation in the Microbiotherid marsupial *Dromiciops gliroides*: Does photoperiod play a role? *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, *203*, 233–240. https://doi.org/10.1016/j.cbpa.2016.09.025

Gannon, W. L., Sikes, R. S., & Comm, A. C. U. (2007). Guidelines of the American Society of Mammalogists for the use of wild mammals in research. *Journal of Mammalogy*, *88*(3), 809–823. https://doi.org/10.1644/06-mamm-f-185r1.1

Garcia-Martinez, J. M., & Alessi, D. R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochemical Journal*, *416*, 375–385. https://doi.org/10.1042/bj20081668

Geiser, F., & Martin, G. M. (2013). Torpor in the Patagonian opossum (*Lestodelphys halli*): Implications for the evolution of daily torpor and hibernation. *Naturwissenschaften*, *100*(10), 975–981. https://doi.org/10.1007/s00114-013-1098-2

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., … Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883

Graves, J. A. M., & Renfree, M. B. (2013). Marsupials in the age of genomics. In A. Chakravarti & E. Green (Eds.), *Annual review of genomics and human genetics* (Vol. 14, pp. 393–420). Palo Alto, CA: Annual Reviews.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: Quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), 1072–1075. https://doi.org/10.1093/bioinformatics/btt086

Habel, J. C., Assman, T., Schmidtt, T., & Avise, J. C. (2010). Relict species: From past to future. In J. C. Habel & T. Assman (Eds.), *Relict species. Phylogeography and conservation biology* (pp. 1–5). Berlin, Germany: Springer.

Hadj-Moussa, H., Moggridge, J. A., Luu, B. E., Quiuntero-Galvis, J. F., Gaitan-Espitia, J. D., Nespolo, R. F., & Storey, K. B. (2016). The hibernating South American marsupial, *Dromiciops gliroides*, display torpor-sensitive microRNA expression patterns. *Scientific Reports*, *6*, 1–11. https://doi.org/10.1038/srep24627

Hand, L. E., Saer, B. R. C., Hui, S. T., Jinnah, H. A., Steinlechner, S., Loudon, A. S. I., & Bechtold, D. A. (2013). Induction of the metabolic regulator Txnip in fasting-induced and natural torpor. *Endocrinology*, *154*(6), 2081–2091. https://doi.org/10.1210/en.2012-2051

Harris, R. A., Bowker-Kinley, M. M., Huang, B. L., & Wu, P. F. (2002). Regulation of the activity of the pyruvate dehydrogenase complex. In G. Weber (Ed.), *Advances in enzyme regulation, Vol 42, Proceedings* (pp. 249–259). Oxford, UK: Pergamon-Elsevier Science Ltd.

Hershkovitz, P. (1999). *Dromiciops gliroides* Thomas, 1894, Last of the Microbiotheria (Marsupialia), with a review of the family Microbiotheridae. *Fieldana*, *93*, 1–60.

Hindle, A. G., Karimpour-Fard, A., Epperson, L. E., Hunter, L. E., & Martin, S. L. (2011). Skeletal muscle proteomics: Carbohydrate metabolism oscillates with seasonal and torpor-arousal physiology of hibernation. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, *301*(5), R1440–R1452. https://doi.org/10.1152/ajpregu.00298.2011

Il'ina, T. N., Ilyukha, V. A., Baishnikova, I. V., Belkin, V. V., Sergina, S. N., & Antonova, E. P. (2017). Antioxidant defense system in tissues of semiaquatic mammals. *Journal of Evolutionary Biochemistry and Physiology*, *53*(4), 282–288. https://doi.org/10.1134/S0022093017040044

Jastroch, M., Giroud, S., Barrett, P., Geiser, F., Heldmaier, G., & Herwig, A. (2016). Seasonal control of mammalian energy balance: Recent advances in the understanding of daily torpor and hibernation. *Journal of Neuroendocrinology*, *28*(11), 10. https://doi.org/10.1111/jne.12437

Jastroch, M., Wuertz, S., Kloas, W., & Klingenspor, M. (2005). Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis. *Physiological Genomics*, *22*(2), 150–156. https://doi.org/10.1152/physiolgenomics.00070.2005

Jiao, Y., Lu, Y., & Li, X. Y. (2015). Farnesoid X receptor: A master regulator of hepatic triglyceride and glucose homeostasis. *Acta Pharmacologica Sinica*, *36*(1), 44–50. https://doi.org/10.1038/aps.2014.116

Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W. Z., McAnulla, C., … Hunter, S. (2014). InterProScan 5: Genome-scale protein function classification. *Bioinformatics*, *30*(9), 1236–1240. https://doi.org/10.1093/bioinformatics/btu031

Koteja, P. (2004). The evolution of concepts on the evolution of endothermy in birds and mammals. *Physiological and Biochemical Zoology*, *77*, 1043–1050. https://doi.org/10.1086/423741

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., … Marra, M. A. (2009). Circos: An information aesthetic for comparative genomics. *Genome Research*, *19*(9), 1639–1645. https://doi.org/10.1101/gr.092759.109

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, *12*, 16. https://doi.org/10.1186/1471-2105-12-323

Liu, M. M., Farkas, M., Spinnhirny, P., Pevet, P., Pierce, E., Hicks, D., & Zack, D. J. (2017). De novo assembly and annotation of the retinal transcriptome for the Nile grass rat (*Arvicanthis ansorgei*). *PLoS One*, *12*(7), 14. https://doi.org/10.1371/journal.pone.0179061

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 38. https://doi.org/10.1186/s13059-014-0550-8

Luu, B. E., Wijenayake, S., Zhang, J., Tessier, S. N., Quintero-Galvis, J. F., Gaitan-Espitia, J. D., … Storey, J. M. (2018a). Strategies of biochemical adaptation for hibernation in a south American marsupial, *Dromiciops gliroides*: 2. Control of the Akt pathway and protein translation machinery. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *224*, 19–25. https://doi.org/10.1016/j.cbpb.2017.12.006

Luu, B. E., Wijenayake, S., Zhang, J., Tessier, S. N., Quintero-Galvis, J. F., Gaitan-Espitia, J. D., … Storey, K. B. (2018b). Strategies of biochemical adaptation for hibernation in a south American marsupial, *Dromiciops gliroides*: 3. Activation of pro-survival response pathways. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *224*, 26–31. https://doi.org/10.1016/j.cbpb.2017.12.005

Malan, A. (2010). Is the torpor-arousal cycle of hibernation controlled by a non-temperature-compensated circadian clock? *Journal of Biological Rhythms*, *25*(3), 166–175. https://doi.org/10.1177/0748730410368621

Maulik, N., & Das, D. K. (2008). Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions. *Biochimica Et Biophysica Acta-General Subjects*, *1780*(11), 1368–1382. https://doi.org/10.1016/j.bbagen.2007.12.008

Mitchell, K. J., Pratt, R. C., Watson, L. N., Gibb, G. C., Llamas, B., Kasper, M., … Cooper, A. (2014). Molecular phylogeny, biogeography, and habitat preference evolution of marsupials. *Molecular Biology and Evolution*, *31*(9), 2322–2330. https://doi.org/10.1093/molbev/msu176

Morin, P., & Storey, K. B. (2009). Mammalian hibernation: Differential gene expression and novel application of epigenetic controls. *International Journal of Developmental Biology*, *53*(2–3), 433–442. https://doi.org/10.1387/ijdb.082643pm

Moriya, Y., Itoh, M., Okuda, S., Yoshizawa, A. C., & Kanehisa, M. (2007). KAAS: An automatic genome annotation and pathway reconstruction server. *Nucleic Acids Research*, *35*, W182–W185. https://doi.org/10.1093/nar/gkm321

Morris, K. M., Weaver, H. J., O'Meally, D., Desclozeaux, M., Gillett, A., & Polkinghorne, A. (2018). Transcriptome sequencing of the long-nosed bandicoot (*Perameles nasuta*) reveals conservation and innovation of immune genes in the marsupial order Peramelemorphia. *Immunogenetics*, *70*(5), 327–336. https://doi.org/10.1007/s00251-017-1043-1

Nelson, C. J., Otis, J. P., & Carey, H. V. (2009). A role for nuclear receptors in mammalian hibernation. *Journal of Physiology-London*, *587*(9), 1863–1870. https://doi.org/10.1113/jphysiol.2008.167692

Nespolo, R. F., Bacigalupe, L. D., Figueroa, C. C., Koteja, P., & Opazo, J. C. (2011). Using new tools to solve an old problem: The evolution of endothermy in vertebrates. *Trends in Ecology & Evolution*, *26*(8), 414–423. https://doi.org/10.1016/j.tree.2011.04.004

Nespolo, R. F., Verdugo, C., Cortes, P. A., & Bacigalupe, L. D. (2010). Bioenergetics of torpor in the Microbiotherid marsupial, Monito del Monte (*Dromiciops gliroides*): The role of temperature and food availability. *Journal of Comparative Physiology B-Biochemical Systemic and*

Environmental Physiology, *180*(5), 767–773. https://doi.org/10.1007/s00360-010-0449-y

Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., … Yodoi, J. (1999). Identification of thioredoxin-binding protein-2/vitamin D-3 up-regulated protein 1 as a negative regulator of thioredoxin function and expression. *Journal of Biological Chemistry*, *274*(31), 21645–21650. https://doi.org/10.1074/jbc.274.31.21645

Opazo, J. C., Nespolo, R. F., & Bozinovic, F. (1999). Arousal from torpor in the chilean mouse-opposum (*Thylamys elegans*): Does nonshivering thermogenesis play a role? *Comparative Biochemistry and Physiology A*, *123*, 393–397. https://doi.org/10.1016/S1095-6433(99)00081-1

Palma, R. E., & Spotorno, A. E. (1999). Molecular systematics of marsupials based on the rRNA 12S mitochondrial gene: The phylogeny of didelphimorphia and of the living fossil microbiotheriid *Dromiciops gliroides* Thomas. *Molecular Phylogenetics and Evolution*, *13*(3), 525–535. https://doi.org/10.1006/mpev.1999.0678

Platani, M., Samejima, I., Samejima, K., Kanemaki, M. T., & Earnshaw, W. C. (2018). Seh1 targets GATOR2 and Nup153 to mitotic chromosomes. *Journal of Cell Science*, *131*, jcs213140. https://doi.org/10.1242/jcs.213140

Renfree, M. B. (1981). Marsupials: Alternative mammals. *Nature*, *293*, 100–101. https://doi.org/10.1038/293100a0

Rose, J. C., Epperson, L. E., Carey, H. V., & Martin, S. L. (2011). Seasonal liver protein differences in a hibernator revealed by quantitative proteomics using whole animal isotopic labeling. *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, *6*(2), 163–170. https://doi.org/10.1016/j.cbd.2011.02.003

Rose, R. W., West, A. K., Ye, J. M., McCormack, G. H., & Colquhoun, E. Q. (1999). Nonshivering thermogenesis in a marsupial (the Tasmanian bettong *Bettongia gaimardi*) is not attributable to brown adipose tissue. *Physiological and Biochemical Zoology*, *72*(6), 699–704. https://doi.org/10.1086/316709

Rouble, A. N., & Storey, K. B. (2015). Characterization of the SIRT family of NAW-dependent protein deacetylases in the context of a mammalian model of hibernation, the thirteen-lined ground squirrel. *Cryobiology*, *71*(2), 334–343. https://doi.org/10.1016/j.cryobiol.2015.08.009

Rouble, A. N., Tessier, S. N., & Storey, K. B. (2014). Characterization of adipocyte stress response pathways during hibernation in thirteen-lined ground squirrels. *Molecular and Cellular Biochemistry*, *393*(1–2), 271–282. https://doi.org/10.1007/s11010-014-2070-y

Ruf, T., & Geiser, F. (2015). Daily torpor and hibernation in birds and mammals. *Biological Reviews*, *90*(3), 891–926. https://doi.org/10.1111/brv.12137

Schwartz, C., Hampton, M., & Andrews, M. T. (2013). Seasonal and regional differences in gene expression in the brain of a hibernating mammal. *PLoS One*, *8*(3), e58427. https://doi.org/10.1371/journal.pone.0058427

Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., & Zdobnov, E. M. (2015). BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*, *31*(19), 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Spindel, O. N., World, C., & Berk, B. C. (2012). Thioredoxin interacting protein: Redox dependent and independent regulatory mechanisms. *Antioxidants & Redox Signaling*, *16*(6), 587–596. https://doi.org/10.1089/ars.2011.4137

Storey, K. B., & Storey, J. M. (2010). Metabolic rate depression: The biochemistry of mammalian hibernation. In G. S. Makowski (Ed.), *Advances on clinical chemistry* (Vol. 52, pp. 77–108). Amsterdam, the Netherlands: Elsevier.

Travisano, M., & Shaw, R. G. (2013). Lost in the map. *Evolution*, *67*(2), 305–314. https://doi.org/10.1111/j.1558-5646.2012.01802.x

Turner, J. M., Warnecke, L., Kortner, G., & Geiser, F. (2012). Opportunistic hibernation by a free-ranging marsupial. *Journal of Zoology*, *286*(4), 277–284. https://doi.org/10.1111/j.1469-7998.2011.00877.x

Vermillion, K. L., Anderson, K. J., Hampton, M., & Andrews, M. T. (2015). Gene expression changes controlling distinct adaptations in the heart and skeletal muscle of a hibernating mammal. *Physiological Genomics*, *47*(3), 58–74. https://doi.org/10.1152/physiolgenomics.00108.2014

Villanueva-Canas, J. L., Faherty, S. L., Yoder, A. D., & Alba, M. M. (2014). Comparative genomics of mammalian hibernators using gene networks. *Integrative and Comparative Biology*, *54*(3), 452–462. https://doi.org/10.1093/icb/icu048

Villarin, J. J., Schaeffer, P. J., Markle, R. A., & Lindstedt, S. L. (2003). Chronic cold exposure increases liver oxidative capacity in the marsupial *Monodelphis domestica*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, *136*(3), 621–630.

Wijenayake, S., Luu, B. E., Zhang, J., Tessier, S. N., Quintero-Galvis, J. F., Gaitan-Espitia, J. D., … Storey, K. B. (2018a). Strategies of biochemical adaptation for hibernation in a south American marsupial *Dromiciops gliroides*: 1. Mitogen-activated protein kinases and the cell stress response. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *224*, 12–18. https://doi.org/10.1016/j.cbpb.2017.12.007

Wijenayake, S., Luu, B. E., Zhang, J., Tessier, S. N., Quintero-Galvis, J. F., Gaitan-Espitia, J. D., … Storey, K. B. (2018b). Strategies of biochemical adaptation for hibernation in a South American marsupial, *Dromiciops gliroides*: 4. Regulation of pyruvate dehydrogenase complex and metabolic fuel selection. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *224*, 32–37.

Wijenayake, S., Tessier, S. N., & Storey, K. B. (2017). Regulation of pyruvate dehydrogenase (PDH) in the hibernating ground squirrel, (*Ictidomys tridecemlineatus*). *Journal of Thermal Biology*, *69*, 199–205. https://doi.org/10.1016/j.jtherbio.2017.07.010

Williams, C. R., Baccarella, A., Parrish, J. Z., & Kim, C. C. (2016). Trimming of sequence reads alters RNA-Seq gene expression estimates. *BMC Bioinformatics*, *17*, 13. https://doi.org/10.1186/s12859-016-0956-2

Wollam, J., & Antebi, A. (2011). Sterol regulation of metabolism, homeostasis, and development. In R. D. Kornberg, C. R. H. Raetz, J. E. Rothman, & J. W. Thorner (Eds.), *Annual review of biochemistry* (Vol. 80, pp. 885–916). Palo Alto, CA: Annual Reviews.

Yoshioka, J., & Lee, R. T. (2014). Thioredoxin-interacting protein and myocardial mitochondrial function in ischemia-reperfusion injury. *Trends in Cardiovascular Medicine*, *24*(2), 75–80. https://doi.org/10.1016/j.tcm.2013.06.007

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biology*, *11*(2), 12. https://doi.org/10.1186/gb-2010-11-2-r14

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Nespolo RF, Gaitan-Espitia JD, Quintero-Galvis JF, et al. A functional transcriptomic analysis in the relict marsupial *Dromiciops gliroides* reveals adaptive regulation of protective functions during hibernation. *Mol Ecol*. 2018;00:1–12. https://doi.org/10.1111/mec.14876