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Ambient temperature-mediated changes in hepatic gene expression of a mammalian herbivore (*Neotoma lepida*)

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Abstract

Herbivores regularly ingest natural toxins produced by plants as a defence against herbivory. Recent work suggests that compound toxicity is exacerbated at higher ambient temperatures. This phenomenon, known as temperature-dependent toxicity (TDT), is the likely result of decreased liver function at warmer temperatures; however, the underlying cause of TDT remains speculative. In the present study, we compared the effects of temperature and dietary plant toxins on differential gene expression in the liver of an herbivorous rodent (*Neotoma lepida*), using species-specific microarrays. Expression profiles revealed a greater number of differentially expressed genes at an ambient temperature below the thermal neutral zone for *N. lepida* (22°C) compared to one within (27°C). Genes and pathways upregulated at 22°C were related to growth and biosynthesis, whereas those upregulated at 27°C were associated with gluconeogenesis, apoptosis and protein misfolding, suggestive of a stressed state for the liver. Additionally, few genes associated with xenobiotic metabolism were induced when woodrats ingested plant toxins compared to non-toxic diets, regardless of temperature. Taken together, the results highlight the important role of ambient temperature on gene expression profiles in the desert woodrat. Temperatures just below the thermal neutral zone might be a favourable state for liver metabolism. Furthermore, the reduction in the number of genes expressed at a temperature within the thermal neutral zone indicates that liver function may be reduced at temperatures that are not typically considered as thermally stressful. Understanding how herbivorous mammals will respond to ambient temperature is imperative to accurately predict the impacts of climate change.

KEYWORDS
endotherms, liver, microarray, plant secondary compounds, plant–animal interactions, temperature-dependent toxicity

1 | INTRODUCTION

Herbivores confront physiological challenges at every meal; plant material is low in nutrients and high in potentially toxic defence compounds (Dearing, Foley, & McLean, 2005; Freeland & Janzen, 1974; Marsh, Wallis, Andrew, & Foley, 2006). Although not traditionally included in theories of diet selection (Raubenheimer, Simpson, & Mayntz, 2009; Stephens & Krebs, 1986), ambient temperature has been demonstrated to have a substantial impact on mammalian herbivores (Belovsky, 1981; Dearing, Forbey, McLister, & Santos, 2008; Owen-Smith, 1998). Work in pharmacology, agriculture and, more recently, ecology has collectively provided support for a phenomenon coined temperature-dependent toxicity (TDT; Dearing, 2013; Keplinger, Lanier, & Deichmann, 1959; Spiers, Evans, & Rottinghaus, 2008). Multiple studies have shown that higher temperatures interact with mammalian physiology in a way that results

in increased toxicity of foreign compounds (Aldrich, Paterson, Tate, & Kerley, 1992; Dearing, 2013; Gordon, 1993; Keplinger et al., 1959). Evidence of TDT has been documented in laboratory rats (Gordon, 1993; Gordon et al., 2008), cattle (Aldrich et al., 1992; Spiers et al., 2008), birds (Chatelain, Halpin, & Rowe, 2013) and woodrats (Dearing et al., 2008; Kurnath & Dearing, 2013). When provided with temperature options, animals challenged with toxins chose cooler ambient temperatures (Gordon et al., 2008) and ingest greater proportions of nontoxic diets at warmer temperatures (Chatelain et al., 2013; Dearing et al., 2008). Furthermore, higher temperatures reduce drug clearance times in both laboratory rats and woodrats, which is indicative of decreased liver function (Kaplanski & Ben-Zvi, 1980; Kurnath & Dearing, 2013). Impaired liver function at warmer temperatures could have critical implications for mammalian herbivores that must balance thermoregulation with the detoxification of plant defence compounds.

The specific mechanisms underlying TDT in natural systems warrant additional attention. One possible mode of action resulting in reduced liver function at warmer temperatures could be changes in liver anatomy and physiology, such as organ size or vascularization that alters blood flow to this heat-producing organ, particularly after a longer temperature acclimation time (Hales, Rowell, & King, 1979; Settivari, Evans, Eichen, Rottinghaus, & Spiers, 2008). Additionally, shifts in enzyme function could alter the efficiency of detoxification resulting in an overall decrease in enzymatic activity in the liver when animals are at higher ambient temperatures (Kaplanski & Ben-Zvi, 1980; Pachecka, Kobylińska, Miaskiewicz, & Bicz, 1982). Changes at the genetic level such as differences in gene expression could also be driving the observed patterns of increased compound toxicity after short-term exposures to warmer ambient temperatures (Settivari et al., 2009). These mechanisms are not mutually exclusive and could be acting in concert, ultimately resulting in reduced liver functionality and elevated compound toxicity for mammals at higher temperatures.

Temperature-mediated shifts in hepatic gene expression were previously documented by Settivari et al. (2009) in laboratory rats fed ergot alkaloids, that is, known fungal toxins that cause fescue toxicosis in cattle feeding on grasses infected with *Neotyphodium coenophialum* (Aldrich et al., 1992). In this study, genes associated with xenobiotic metabolism, oxidative phosphorylation and antioxidative mechanisms were downregulated in the liver after a short-term heat shock treatment (Settivari et al., 2009). Conversely, genes associated with gluconeogenesis and apoptosis were upregulated after heat stress, demonstrating that laboratory rats were calorically stressed and experiencing hepatic tissue injury when faced with both high temperatures and foreign toxins (Guicciardi, Malhi, Mott, & Gores, 2013; Settivari et al., 2009). Their work supports the prediction that gene expression could contribute to TDT; however, temperature treatments in this study spanned 10°C, with the higher temperature being a thermally stressful one for laboratory rats (31°C). Heat stress of this magnitude influences hepatic gene expression in the absence of dietary toxins (Feder & Hofmann, 1999; Hochachka & Somero, 2002; Sonna, Fujita, Gaffin, & Lilly, 2002). The effect of temperature differences on hepatic gene expression below the boundaries of heat stress has received little to no attention (e.g., Ueta, Olivares, & Bianco, 2011).

Here, we explored gene expression as a putative mechanism of TDT at nonthermally stressful temperatures in mammalian herbivores, the desert woodrat (*Neotoma lepida*, Figure 1a). Woodrats from the Mojave Desert are well suited for this task because populations feed on creosote bush (*Larrea tridentata*, up to 75% of diet), a highly defended shrub that produces a toxic resin on its leaves (Karasov, 1989). These woodrats can ingest doses of toxins in creosote resin that cause kidney cysts and even death in laboratory rodents (Goodman, Grice, Becking, & Salem, 1970; Karasov, 1989). Gene expression studies previously conducted with *Neotoma* identified candidate hepatic enzymes important for the metabolism of plant toxins, including those in creosote resin (Dearing, Skopec, & Bastiani, 2006; Magnanou, Malenke, & Dearing, 2009, 2013), and documented convergent detoxification strategies used by different woodrat species (Malenke, Milash, Miller, & Dearing, 2013; Malenke, Skopec, & Dearing, 2014).

In our study, we set out to investigate patterns of hepatic gene expression across temperature and toxin treatments in *N. lepida*. First, we determined the effect of temperature (22°C vs. 27°C) on gene expression while animals ingested a control diet (i.e., without a xenobiotic resin extracted from the creosote bush). We predicted overall lower gene expression at warmer temperatures compared to cooler temperatures, based on previous evidence suggesting gene expression and hence liver function were reduced at higher temperatures (Settivari et al., 2008, 2009). Second, we contrasted the effect

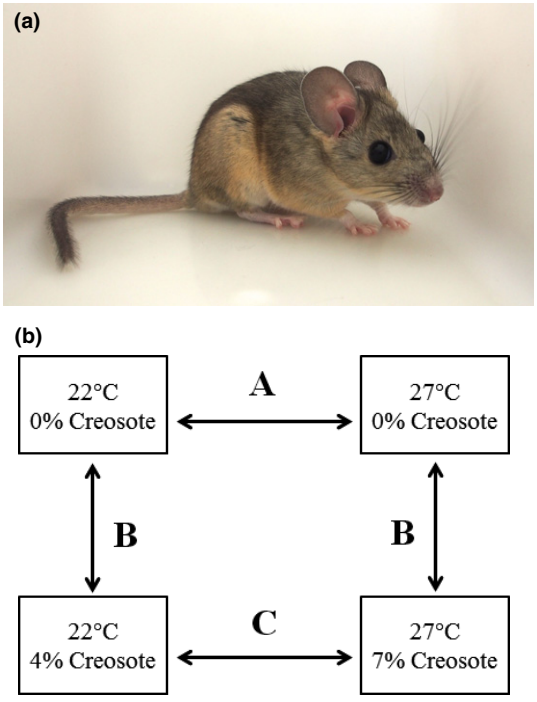

FIGURE 1 (a) *Neotoma lepida*, used with permission of K.D. Kohl. (b) Diagram defining all pairwise comparisons (letters and arrows) and treatment groups (squares) in the current study ($N = 4$ per group) [Colour figure can be viewed at wileyonlinelibrary.com]

of diet (control vs. creosote resin) within a temperature treatment (22 and 27°C). We expected creosote diets to induce detoxification genes and to induce to a greater extent at 22°C compared to 27°C.

2 | MATERIALS AND METHODS

2.1 | Animal collection

Adult desert woodrats, *Neotoma lepida* ($N = 16$, eight males and eight females), were collected from the Mojave Desert near Beaver Dam, Utah (37°06′N, 133°58′W), in May 2012. Sherman live traps were baited with apples, oats and peanut butter, and cotton batting was provided for nesting material. Creosote leaves were collected from several plants (~10) at the trapping sites for the extraction of resin. Plant material was stored on dry ice in the field and at −20°C at the University of Utah (Salt Lake City, UT). Woodrats were transported to an animal facility at the University of Utah and maintained in shoebox cages (48×27x20 cm) at room temperature (23–25°C) on a 12L:12D cycle. Woodrats were provided rabbit chow pellets (Teklad 2031 formula, Harlan Laboratories, Madison, WI) and water ad libitum for at least 3 weeks before experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC #12-12010).

2.2 | Feeding trial

Woodrats were divided evenly into four groups (balanced for sex and body mass) and exposed to four different treatments of temperature and diet. The two temperature treatments used in the current study included one within the thermal neutral zone of *N. lepida* (27°C or thermoneutral) and another below the thermal neutral zone (22°C or cool; thermal neutral zone 25–34°C, Kurnath & Dearing, 2013). Similar temperatures have been used in previous studies (Dearing et al., 2008; Kurnath & Dearing, 2013; Kurnath, Merz, & Dearing, 2016; McLister, Sorensen, & Dearing, 2004), and these temperatures are ecologically relevant for Mojave Desert woodrats (Kurnath & Dearing, 2013). Room temperatures were measured every 10 min with HOBO data loggers (UA-001-08 pendant, Onset, Bourne, MA) prior to and during experiments. Animals were acclimated to either 27°C or 22°C for at least 14 days prior to experiments similar to previous studies (Dearing et al., 2008; Kurnath et al., 2016; McLister et al., 2004).

At each temperature, animals were presented one of two diet treatments, either powdered rabbit chow (Harlan Laboratories) without creosote resin (i.e., control treatment) or powdered rabbit chow amended with creosote resin (i.e., creosote treatment). Resin was extracted from foliage and diets were prepared as in Magnanou et al. (2009). Animals were fed a gradually increasing amount of resin over an 8-day period to allow induction of biotransformation enzymes (Klaassen, 2001). Based on previous work, we expected different daily food intakes by animals at the two temperatures (Kurnath et al., 2016); we therefore a priori altered the resin concentrations of the diets to ensure all animals ingested on average 0.3 g of creosote resin per day. At the cool temperature, animals in the creosote treatment received powdered rabbit chow with 0% resin for 4 days, 2% resin for 2 days, followed by 4% resin for the final 2 days. At the thermoneutral temperature, animals in the creosote treatment received powdered rabbit chow with 0% resin for 4 days, 3.5% resin for 2 days, followed by 7% resin for the final 2 days. Final doses of creosote resin were comparable to those found in the natural diet of *N. lepida* in the wild, which can be up to 7% resin (Cameron & Rainey, 1972; Karasov, 1989; Meyer & Karasov, 1989). In this study, the animals did not differ significantly in food intake, and, as a result, the resin intakes differed across temperature treatments, with the animals in the thermoneutral temperature consuming 50% more creosote resin per gram body mass than animals in the cool (Table 1). Animals in the control treatments received powdered rabbit chow with 0% resin for all 8 days of the trial. Woodrats had ad libitum access to water and food during the feeding trial.

Food intake, creosote resin intake and body mass were measured daily. Mean body mass and food intake per gram body mass from the final 2 days of the trial were compared across all four treatment groups using two-way ANOVAs with post hoc Tukey's HSD tests, with diet and temperature as independent variables. Mean resin intake per gram body mass from the last 2 days of the trial was compared across temperature groups using a one-way ANOVA. All data were normally distributed.

At the end of the feeding trial, animals were dispatched using CO_2 asphyxiation. Liver weight was measured and compared across groups with a two-way ANOVA and post hoc Tukey's HSD test, with diet and temperature as independent variables. Liver tissue was preserved in RNAlater (Sigma, St. Louis MO) and archived at −80°C, as per product instructions. RNA was extracted from the samples and treated with DNase Inactivation reagent (RNAqueous, Applied Biosystems, Foster City, CA) and sent to the University of Utah Microarray Core Facility (Huntsman Cancer Institute, Salt Lake City, UT).

2.3 | Microarray quality assurance

Liver samples were analysed on a custom microarray platform built based on a liver transcriptome (Roche 454 platform) of the desert woodrat (Malenke et al., 2013). *Neotoma*-specific transcripts were annotated with multiple rodent species, the vast majority based upon *Rattus norvegicus* and *Mus musculus*. Details on the design of the custom microarray (eArray, Agilent, Santa Clara, CA) and processing methods can be found elsewhere (Malenke et al., 2013, 2014). Each RNA sample was hybridized to a separate array, resulting in four biological replicates for each temperature and diet combination. All raw microarray data were deposited in Gene Expression Omnibus (NCBI, series record GSE80595) in accordance with minimum information about microarray experiment compliance or MIAME.

Microarray features were extracted using AGILENTFILTER software (BioInformatic Shared Resource, Huntsman Cancer Institute and University of Utah, Salt Lake City, UT) as previously described

TABLE 1 Comparisons of data collected during 8-day feeding trial across four experimental treatments of woodrats (N = 16 in total, N = 4 per treatment group)

	Diet treatment		Temperature		Diet		Interaction	
	Control	Creosote	*F*	*p*	*F*	*p*	*F*	*p*
Food intake (g) per gram body mass								
22°C	0.0709 ± 0.006	0.0574 ± 0.006	1.2	.30	5.0	**.05**	0.0	.95
27°C	0.0642 ± 0.005	0.0514 ± 0.006						
Resin intake (g) per gram body mass								
22°C	n/a	0.0023 ± 0.0002	7.5	**.03**	n/a	n/a	n/a	n/a
27°C	n/a	0.0036 ± 0.0004						
Starting body mass (g)								
22°C	124.7 ± 8.0	124.0 ± 11.0	0.1	.78	0.0	.87	0.0	.93
27°C	128.6 ± 12.5	126.0 ± 8.1						
Ending body mass (g)								
22°C	123.4 ± 7.2	125.5 ± 10.2	0.1	.81	0.0	.88	0.1	.72
27°C	129.3 ± 12.8	124.5 ± 7.9						
Liver mass (g)								
22°C	3.6 ± 0.2	4.1 ± 0.5	1.6	.24	0.3	.63	0.9	.37
27°C	3.5 ± 0.5	3.3 ± 0.4						
Liver mass, relative to body mass (%)								
22°C	3.0 + 0.1	3.4 + 0.3	4.5	.06	0.6	.45	1.3	.29
27°C	2.7 + 0.2	2.7 + 0.3						

All comparisons are two-way ANOVAs (df = 1) with the exception of the variable Resin Intake, which is a one-way ANOVA. Results are recorded as means ± *SEM* values. p values of ≤.05 are bolded.

(Malenke et al., 2013, 2014). Briefly, all control spots, nonuniform spots and population outlier spots were removed from the data set and intensity values were $\log_2$-transformed. Intensity data from duplicate probes were combined into unique probe groups. Spot intensity data from different probes with the same annotation information were maintained separately because it is possible that the original assembled contigs, from which the probes were designed, were from different genetic loci despite the shared match to a single rodent gene annotation.

2.4 | Comparisons of gene expression

Spot intensity data from the *Neotoma* microarray were batch-uploaded to GENESIFTER 3.7 (Geospiza, PerkinElmer, Seattle, WA). Prior to comparing expression profiles, the consistency of transcriptome response was evaluated by comparing overall gene expression profiles across individuals. After normalizing intensity data, individuals were clustered by gene expression using all woodrat-derived probes (N = 6,286). Clustering parameters were distance:correlation, linkage: average and row centred:by genes.

Multiple pairwise comparisons were generated in GENESIFTER to compare gene expression. In all comparisons, the quality requirement was set to 1 and all individuals from both treatments were required to pass. *T*-tests were performed (α = 0.05) and the resulting gene/probe lists were ranked by fold change (≥2). This method is less conservative than applying a false discovery rate correction (Benjamini & Hochberg, 1995), yet fold change ranking is more consistent (Guo et al., 2006).

A total of four pairwise comparisons were made in GENESIFTER (Figure 1b). To investigate the effect of temperature, animals at 27°C were compared to animals at 22°C while ingesting control diets. Transcripts were described as upregulated at either the thermoneutral (27°C) or the cool (22°C) temperature. We ran two comparisons to investigate the effect of diet: one comparison with animals at 22°C and another comparison with animals at 27°C. Within a temperature treatment, animals feeding on the creosote diet were compared to animals fed the control diet. Transcripts that were upregulated on the creosote diet compared to the control diet were considered genes induced by creosote resin. Lastly, animals at 27°C were compared to animals at 22°C while ingesting diets with creosote resin. The lists of significantly different genes were ordered by fold change and direction.

Treatments were compared by examining the number of annotated, upregulated transcripts as well as the KEGG pathways and Gene Ontology (GO) terms for biological processes represented by those transcripts. *z*-scores were used to determine significant representation in these functional assays. KEGG pathways or GO terms with *z*-scores >2 were considered to be significantly overrepresented in the results. The number of transcripts, fold change values and annotation of the hybridized probe were reported for each comparison. We also identified and categorized transcripts based on potential function from GO terms and KEGG pathways.

3 | RESULTS

3.1 | Feeding trial

Diet significantly affected total food intake; woodrats fed control diets always ate more food than woodrats fed creosote diets, regardless of temperature. There was no effect of temperature, nor an interaction effect on food intake (Table 1). Animals at 27°C ingested ~11% less food on both diets compared to woodrats at 22°C, but this difference was not significant. There was no effect of temperature or diet on starting or ending body mass (Table 1), and all woodrats maintained body mass throughout the 8-day feeding trial (paired *t*-test, $p = .8$). Similarly, there was no effect of temperature or diet on liver mass of woodrats, even though average liver size at 22°C was 14% larger in animals ingesting the creosote diet compared to animals ingesting the control diet (Table 1).

3.2 | Microarray quality assurance

The microarray was previously validated (Malenke et al., 2013, 2014). All of the 16 arrays passed at least six of Agilent's nine quality metrics. Three samples passed seven metrics, nine samples passed eight metrics, and three samples passed all nine quality metrics. All samples were kept in the analysis. On average, less than 0.05% of the features across all arrays were flagged as nonuniform. The grouping of individual woodrats by overall expression profile placed woodrats from the "cool, creosote" treatment in one group (Figure 2). The remaining individuals did not group by treatment but rather by temperature. Woodrats at 27°C generally grouped together regardless of dietary treatment.

3.3 | Overall transcript expression

A total of 107 unique transcripts were differentially expressed across all four pairwise comparisons made in this study. Of these results, only 10 transcripts appeared in more than one comparison (Fig. S1). Two of these 10 transcripts were associated with detoxification (p450 oxidoreductase and glutathione S-transferase mu 2) and two transcripts were associated with hepatocellular carcinomas (annexin A3 and histidine triad nucleotide binding protein 2). Multiple transcripts appear conserved in their response, particularly at 22°C. For instance, three transcripts were upregulated at 22°C while animals were feeding on creosote diets and were induced by creosote while animals were housed at 22°C (Fig. S1, points with green outline and blue fill, respectively). One transcript in this cluster, annexin A3, displayed the greatest fold change (4.5×, induced on creosote diet compared to control diet at 22°C).

3.4 | Transcripts influenced by temperature

Temperature influenced differential gene expression in woodrats ingesting a control diet (Figure 1b, comparison A). On the control diet, 15 transcripts were significantly upregulated at 22°C while only

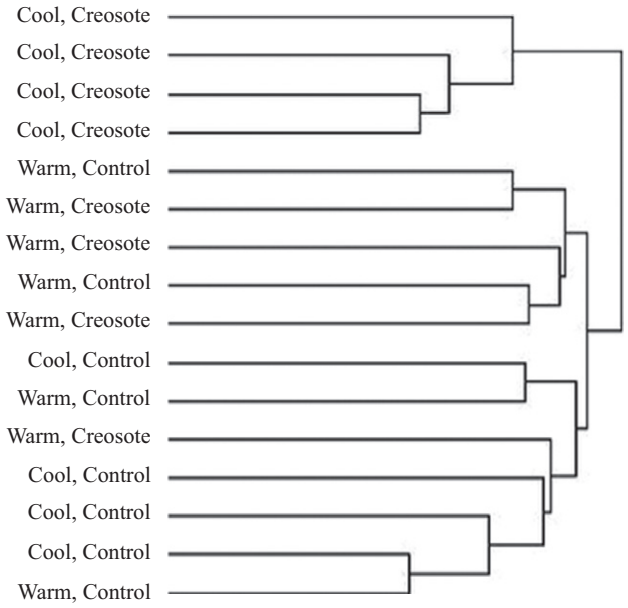

FIGURE 2 Cluster analysis of individual woodrat samples calculated from overall gene expression profiles. Individuals are specified by temperature (cool = 22°C, warm = 27°C) and diet treatments (control, creosote)

eight transcripts were significantly upregulated at 27°C (Table 2). In addition, more transcripts associated with detoxification genes were differentially expressed at 22°C compared to 27°C (three vs. 0 transcripts, Table 2). A transcript variant of Tsukushi displayed the highest fold change at 22°C (Tsku, 5.4×), and a member of the tumour necrosis factor receptor superfamily had the highest fold change at 27°C (Tnfsrf18, 2.9×; Table 3).

Genes upregulated at 22°C had different functions compared to genes upregulated at 27°C. Genes involved in repair and regrowth were upregulated at 22°C, including DNA repair, steroid biosynthesis, metabolism and organ regeneration. Oxidation–reduction reactions, cell adhesion and signalling were other molecular processes notably upregulated at 22°C. Conversely, at least two genes significantly upregulated at 27°C were involved in cell death and degradation: Tnfsrf10b (apoptosis) and Snx16 (targeting for lysosome). Other potential functions of genes upregulated at 27°C include binding, ER and extracellular region, transferase activity and signal transduction.

More KEGG pathways were overrepresented in animals eating a control diet at 27°C compared to 22°C (14 vs. eight pathways; Table 4). Most pathways at 22°C were associated with a single transcript for bile acid coenzyme A (Baat, 5 pathways), while at 27°C most pathways were associated with one transcript with detoxifying function, UDP glucuronosyltransferase (Ugt2b34, 10 pathways). Regardless of temperature, 16 of 22 KEGG pathways listed were associated with metabolism. No GO terms contained gene lists with >10 transcripts.

Temperature affected transcript numbers in the two comparisons testing the effect of diet (Figure 1b, comparison B). At 22°C, nearly fivefold more transcripts were differentially expressed in a comparison of control and creosote diets than at 27°C (49 vs. 10

TABLE 2 Number of transcripts with significantly different expression for each comparison made in the current study

Comparison	Number of transcripts	
	All	Detox
(A) On control diet		
Higher expression at 27°C	8	2
Higher expression at 22°C	15	4
(B) At 22°C (0% vs. 4% creosote diets)		
Induced expression on creosote	29	1
Repressed expression on creosote	20	2
(B) At 27°C (0% vs. 7% creosote diets)		
Induced expression on creosote	5	0
Repressed expression on creosote	5	0
(C) On creosote diets		
Higher expression at 27°C	15	7
Higher expression at 22°C	20	13

Transcripts associated with detoxification and xenobiotic metabolism are listed.

transcripts, Table 5). Additionally, more KEGG pathways were overrepresented in the 22°C comparison vs. the 27°C comparison (Table 6). The KEGG pathways at 22°C were based upon 15 unique transcripts, whereas only seven unique transcripts were associated with the overrepresented KEGG pathways at 27°C.

3.5 | Transcripts induced and repressed by creosote

Two within-temperature treatment comparisons were made to investigate the impact of diet on hepatic gene expression (Figure 1b, comparison B). One transcript appeared in both comparisons and was induced by creosote in both instances: Cyp26a1 from the cytochrome p450 family that regulates cellular levels of retinoic acid, a metabolite of vitamin A.

At 22°C, 49 transcripts were differentially expressed, with 29 genes induced and 20 genes repressed by a creosote diet (Table 2). Very few differentially expressed genes at 22°C were associated with detoxification; only one induced gene (Gstm2) and two repressed genes (Por and Fmo3; Table 5), all of which also had low fold change values. The transcript with the highest fold change was annexin A3 (4.5×; Table 5), which was induced on creosote and involved in phagocytosis. Most other transcripts induced on the creosote diet were associated with general processes within the cell, including transcripts involved in cell adhesion, cytoskeleton organization and cell migration. Only one transcript pertaining to xenobiotic metabolism (Gstm2) was induced by creosote. In addition, transcription-related processes, protein phosphorylation and the regulation of various enzymatic activities were significantly induced by creosote resin at 22°C. Lastly, creosote resin also induced two immune response transcripts and one associated with apoptosis.

Transcripts related to pain sensory, response to stress, transcription and DNA repair were repressed by creosote at 22°C. There were many processes repressed by creosote with only one associated transcript including the innate immune response, lipid transport, blood coagulation, histone deacetylation, ubiquitin-dependent process, Ras protein signalling and kidney development. Taken together, genes influenced by dietary creosote at 22°C were not associated with detoxification but many other, diverse metabolic processes.

At 22°C, more GO terms were overrepresented by transcripts induced by creosote resin than those repressed by creosote, by a difference of threefold (35 vs. 13 terms; Table S1). Overrepresented KEGG pathways showed a similar pattern, by a difference of sixfold (26 induced pathways vs. four repressed pathways, Table 6). In addition, GO term and KEGG pathways overrepresented due to dietary creosote were associated with xenobiotic metabolism and detoxification. More than half of the KEGG pathways induced by creosote resin were categorized as human disease related (15 pathways), encompassing 12 unique transcripts.

Substantially fewer transcripts were induced and repressed by dietary creosote at 27°C compared to 22°C (Table 2). Only five transcripts were induced, and five transcripts were repressed by creosote diet ingested by *N. lepida* at 27°C. None of the transcripts were involved in xenobiotic metabolism. The highest fold change value belonged to D-site of albumin promoter (Dbp, 5.3×; Table 5), which was repressed on creosote and plays an important role in cell survival, proliferation and differentiation. There were no GO terms that contained gene lists of >10 transcripts. Additionally, significantly fewer KEGG pathways were overrepresented at 27°C compared to 22°C (12 vs. 30 KEGG pathways; Table 6). Most KEGG pathways induced by creosote were associated with carbohydrate metabolism. The majority of transcripts and KEGG pathways resulting from this comparison were associated with gluconeogenesis, glycolysis and glucose/glycogen pathways.

3.6 | Transcripts affected by temperature and creosote

Similar to other comparisons, more differentially expressed transcripts were found to be upregulated at 22°C compared to 27°C in woodrats presented diets amended with creosote resin (Figure 1b, comparison C; Table 2). In addition, this comparison resulted in the greatest number of differentially expressed transcripts associated with detoxification (seven transcripts at 27°C and 13 transcripts at 22°C; Table 7). There were also more GO terms and KEGG pathways significantly overrepresented at 22°C compared to at 27°C (Tables S2 and S3).

4 | DISCUSSION

Although there has been mounting evidence for temperature-dependent toxicity (TDT) in model and nonmodel systems, the underlying mechanism of how toxicity increases at warmer ambient temperatures has not been extensively explored. In this study, we investigated the effect of ambient temperature and dietary toxins on

TABLE 3 Transcripts differentially expressed at 27 and 22°C while woodrats were ingesting diets without creosote resin (comparison A)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
(a) Upregulated at 27°C					
NM_001024349	Tnfrsf18	Tumour necrosis factor receptor superfamily, member 18	Signal transduction, receptor and molecular transducer activity	2.9	.019
NM_008458	Serpina3c	Serine (or cysteine) peptidase inhibitor, clade A, member 3C	Regulation (peptidase activity, endopeptidase activity, proteolysis)	2.9	.028
NM_001038588	**Prodh2**	**Proline dehydrogenase (oxidase) 2**	**Glutamate biosynthetic process, oxidation–reduction process**	**2.6**	**.028**
NM_001107561	Scgb1c1	Secretoglobin, family 1C, member 1	Binding, ER and extracellular region	2.4	.013
NM_153598	**Ugt2b34**	**UDP glucuronosyltransferase 2 family, polypeptide B34**	**Transferase activity, metabolic process**	**2.3**	**.001**
NM_020275	Tnfrsf10b	Tumour necrosis factor receptor superfamily, member 10b	Apoptosis, signal transduction, induction of apoptosis	2.3	.038
NM_029068	Snx16	Sorting nexin 16, transcript variant 1	Targeting for lysosome, transport, cell communication	2.2	.032
NM_001079939	Lmf2	Lipase maturation factor 2	ER membrane, extracellular region	2.1	.002
(b) Upregulated at 22°C					
NM_001168541	Tsku	Tsukushi, transcript variant 1	Signalling	5.4	.001
NM_031768	Itgae	Integrin, alpha E	Cell adhesion, integrin-mediated signalling pathway	5.4	.029
NM_001126273	Alkbh2	alkB, alkylation repair homolog 2 (E. coli)	DNA repair, oxidation–reduction reaction, DNA demethylation	3.5	.005
NM_026871	Hint2	Histidine triad nucleotide binding protein 2	Hepatocellular carcinomas, steroid biosynthesis, apoptosis	2.7	.016
NM_017300	Baat	Bile acid coenzyme A: amino acid *N*-acyltransferase (glycine *N*-choloyltransferase)	Metabolism (lipid, fatty acid, acyl-CoA, bile acid, steroid), acute-phase response, inflammatory response	2.3	.014
NM_001170477	Grtp1	Growth hormone-regulated TBC protein 1, transcript variant 1	Molecular function regulator, regulation of primary metabolic process	2.2	.009
NM_013872	Pmm1	Phosphomannomutase 1, transcript variant 1	Metabolism, dolichol-linked oligosaccharide biosynthetic process	2.2	.005
NM_025459	Fam134b	Family with sequence similarity 134, member B, transcript variant 2	Pain sensory perception, neurological system process	2.2	.045
NM_175507	Slc35 g1	Solute carrier family 35, member G1	Inorganic ion homeostasis, calcium export, cation homeostasis	2.2	.013
NM_010726	**Phyh**	**Phytanoyl-CoA hydroxylase**	**Metabolism, oxidation–reduction reactions**	**2.1**	**.045**
NM_001159626	**Hagh**	**Hydroxyacyl glutathione hydrolase, transcript variant 2**	**Pyruvate metabolism and citric acid cycle, catabolic process**	**2.1**	**.004**
NM_001159626	**Hagh**	**Hydroxyacyl glutathione hydrolase, transcript variant 2**	**Pyruvate metabolism and citric acid cycle, catabolic process**	**2.1**	**.003**
NM_001034912	Fam134b	Family with sequence similarity 134, member B	Pain sensory perception, neurological system process	2.0	.026
NM_199372	Eif4a1	Eukaryotic translation initiation factor 4A1	Organ regeneration, multicellular organismal development, anatomical structure morphogenesis	2.0	.009
NM_008898	**Por**	**P450 (cytochrome) oxidoreductase**	**Regulation of monooxygenase activity, fatty acid oxidation**	**2.0**	**.017**

Bolded transcripts are associated with detoxification of plant secondary compounds. Associated functions compiled by GENESIFTER and supplemented from NCBI Biosystems.

patterns of relative gene expression in the liver of the desert woodrat, *Neotoma lepida*, a homeothermic mammal. We found that differential gene expression in the liver was greatest at ambient temperatures (22°C) slightly below the thermal neutral zone (TNZ) of *N. lepida* than a warmer, thermoneutral ambient temperature (27°C) within the TNZ. These results suggest that small changes in ambient

TABLE 4 KEGG pathways overrepresented at 27 and 22°C in animals ingesting diets without creosote resin (comparison A)

KEGG pathway	List	Gene set	*z*-score
(a) Pathways upregulated at 27°C on control diet			
Cytokine–cytokine receptor interaction	2	32	7.25
Drug metabolism—cytochrome P450	1	66	2.25
Metabolism of xenobiotics by cytochrome P450	1	54	2.56
Retinol metabolism	1	42	2.98
Drug metabolism—other enzymes	1	39	3.11
Arginine and proline metabolism	1	37	3.21
Apoptosis	1	27	3.84
Porphyrin and chlorophyll metabolism	1	26	3.92
Steroid hormone biosynthesis	1	26	3.92
Starch and sucrose metabolism	1	23	4.2
Natural killer cell-mediated cytotoxicity	1	21	4.41
Pentose and glucuronate interconversions	1	21	4.41
Ascorbate and aldarate metabolism	1	19	4.66
Other types of O-glycan biosynthesis	1	15	5.28
(b) Pathways upregulated at 22°C on control diet			
Peroxisome	2	47	5.21
Regulation of actin cytoskeleton	1	54	2.21
Bile secretion	1	28	3.31
Amino sugar and nucleotide sugar metabolism	1	21	3.9
Fructose and mannose metabolism	1	17	4.38
Biosynthesis of unsaturated fatty acids	1	13	5.06
Primary bile acid biosynthesis	1	12	5.28
Taurine and hypotaurine metabolism	1	2	13.27

"List" indicated the number of genes highly expressed within the pathway. "Gene set" indicates the total number of genes in that pathway included on the array.

temperature can result in large differences in hepatic gene expression in a mammalian herbivore, even when diet is held constant. Furthermore, it is critical to recognize that liver gene expression was reduced at nonstressful ambient temperatures that occurred within the TNZ. This result suggests that while the TNZ may be optimal for minimizing energy expenditure, it may not be optimal for the metabolism of dietary toxins. Ambient temperatures just below the TNZ may permit higher detoxification rates by the liver.

4.1 | Greater differential gene expression at cooler ambient temperatures

More transcripts were differentially expressed at 22°C than at 27°C for animals ingesting a nontoxic diet (Figure 1b, comparison A), supporting our prediction that reduced gene expression at warmer temperatures could explain reduced liver function observed in earlier studies (Kaplanski & Ben-Zvi, 1980; Keplinger et al., 1959; Kurnath & Dearing, 2013). A surprising result from this comparison was the numerous KEGG pathways related to detoxification that were overrepresented at 27°C (Table 4). These pathways were mostly associated with a single gene, UDP glucuronosyltransferase (Ugt2b34). This gene is part of a family of conjugating enzymes that is important in *Neotoma* species for metabolizing creosote resin (Haley, Lamb, Franklin, Constance, & Dearing, 2008; Lamb, Sorensen, & Dearing, 2001; Malenke et al., 2014; Mangione, Dearing, & Karasov, 2000, 2001); but the animals in this comparison were not feeding on creosote resin. UGTs are versatile enzymes that can also conjugate endogenous substrates such as steroids and bilirubin (Klaassen, 2001). The upregulation of Ugt2b34 at 27°C compared to 22°C could be indicative of an increased need for endogenous glucuronidation and may be a response to increased steroid biosynthesis, which was also an overrepresented KEGG pathway. The increased need for endogenous glucuronidation at warmer temperatures could in turn reduce the availability of UGTs to perform other functions such as the biotransformation of dietary toxins, which would likely have implications for woodrats ingesting creosote resin at similar temperatures.

Ambient temperature appeared to shift enzymatic functions in the liver, such that gene expression patterns at 22°C indicative of growth functions were replaced with those related to stress responses at 27°C. For instance, genes associated with steroid biosynthesis (histidine triad nucleotide binding protein 2), DNA repair (alkylation repair homolog 2) and metabolism (bile acid coenzyme A: amino acid *N*-acyltransferase) were overrepresented at 22°C compared to 27°C. These genes could be indicative of biosynthesis and may represent a state of healthy protein building for the liver at 22°C. In contrast, genes associated with apoptosis (tumour necrosis factor receptor superfamily 18 and 10b) were significantly overrepresented at 27°C compared to 22°C. Similar patterns of gene expression at warm ambient temperatures have been reported in other studies, regardless of acclimation time to relatively challenging ambient temperatures. Increased apoptotic processes were previously reported in a study testing the effects of short-term heat shock in mice challenged with toxins (Settivari et al., 2009). Tumour necrosis factors have also been described as heat shock-related genes and were upregulated in a study investigating chronic heat exposure in chickens (Li, Wang, Wang, Li, & Wu, 2011). Additionally, protein misfolding genes were upregulated at 27°C compared to 22°C (serine peptidase inhibitor A3C), a pattern previously documented in laboratory rats exposed to short-term heat stress (Stallings et al., 2014). Patterns in gene expression would be expected to differ based on short-term or chronic heat stress because of the often immediate and temporary response at the cellular level to a new environmental factor (Pappenheimer, Fregly, & Blatties, 1996). Yet regardless of acclimation time, higher ambient temperatures appeared to result in a common gene expression response associated with stress. Combined with the overrepresented KEGG pathways involving metabolism and immune system responses, the pattern of upregulated genes noted in the current study implies that 27°C is also a relatively more stressful state than 22°C for woodrats.

TABLE 5 Transcripts differentially expressed by dietary creosote resin compared to control diet (comparison B) at 22°C (a, b) and at 27°C (c, d)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
(a) Induced by creosote at 22°C					
NM_013470	Anxa3	Annexin A3	Hepatocellular carcinomas, cell migration, phagocytosis	4.5	.004
NM_001082548	Spint2	Serine protease inhibitor, Kunitz type 2, transcript variant 2	Regulation of peptidase activity, regulation of hydrolase activity	4.1	.001
NM_001008847	RT1-Da	RT1 class II locus Da	Lysosome, lysosome membrane, regulation of enzymatic activity	3.6	.014
NM_010831	Sik1	Salt-inducible kinase 1	Transcription-related processes, protein phosphorylation, cell cycle	3.3	.047
NM_008361	Il1b	Interleukin 1 beta	Protein phosphorylation, activation of MAPK activity, fever generation	3.2	.023
NM_001109011	Marco	Macrophage receptor with collagenous structure	Scavenger receptor activity, transmembrane receptor activity	3.0	.034
NM_007811	Cyp26a1*	Cytochrome P450, family 26, subfamily a, polypeptide 1	Retinoic acid catabolic and metabolic processes, central nervous system development	2.8	.040
NM_001168633	Irs2	Insulin receptor substrate 2	Signalling (RET, growth hormone receptor, interleukin receptor)	2.8	.022
NM_008538	Marcks	Myristoylated alanine-rich protein kinase C substrate	Binding (actin, protein kinase C, calmodulin, cytoskeleton protein)	2.8	.011
NM_130408	Cyp26a1*	Cytochrome P450, family 26, subfamily a, polypeptide 1 (rat)	Retinoic acid catabolic and metabolic processes, central nervous system development	2.7	.036
NM_001009353	Pla2 g7	Phospholipase A2 group VII (platelet-activating factor acetylhydrolase plasma)	Regulation of enzymatic activity, primary metabolic process, lipid catabolic process	2.7	.015
NM_133416	Bcl2a1d	B-cell leukaemia/lymphoma 2-related protein A1	Apoptosis, regulation of apoptosis, B-cell homeostasis, cellular process	2.7	.032
NM_013566	Itgb7	Integrin, beta 7	Cell adhesion, integrin-mediated signalling pathway	2.5	.024
NM_010693	Lck	Lymphocyte protein tyrosine kinase, transcript variant 2	Protein phosphorylation, induction of apoptosis, activation of caspase activity	2.5	.002
NM_017196	Aif1	Allograft inflammatory factor 1	Protein phosphorylation, regulation (gene expression, cell migration)	2.4	.024
NM_010545	Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex class II antigen-associated), transcript variant 2	Activation of MAPK activity, prostaglandin biosynthetic process, regulation of cytokine-mediated signalling pathway	2.4	.016
NM_008788	Pcolce	Procollagen C-endopeptidase enhancer protein	Regulation of enzymatic activity, proteolysis, peptidase activity regulation	2.3	.025
NM_053018	Cd9	CD9 molecule	Cell adhesion, cellular component movement	2.2	.008
NM_021278	Tmsb4x	Thymosin, beta 4, X chromosome	Cytoskeleton organization, cell migration regulation	2.2	.029
NM_001161845	Sgk1	Serum/glucocorticoid-regulated kinase 1, transcript variant 1	Ion channel regulator activity, stimuli-sensing channel, transmembrane transport	2.2	.037
NM_019337	Rgs10	Regulator of G-protein signalling 10	Regulation of G-protein-coupled receptor protein signalling pathway	2.2	.005
NM_010745	Ly86	Lymphocyte antigen 86	Innate immune response, inflammatory response	2.1	.048
NM_008026	Fli1	Friend leukaemia integration 1	Transcription-related processes (DNA dependent), transcription regulation, blood circulation	2.1	.018

(Continues)

TABLE 5 (Continued)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
NM_007908	Eef2k	Eukaryotic elongation factor-2 kinase, transcript variant 1	Protein phosphorylation, phosphate metabolic process	2.1	.035
NM_009254	Serpinb6a	Serine (or cysteine) peptidase inhibitor, clade B, member 6a, transcript variant 2	Regulation of enzymatic activity (peptidase, endopeptidase, hydrolase, proteolysis and catalytic activity)	2.1	.029
XM_573272	Hmgb2l1	PREDICTED: High-mobility group box 2-like 1	Transcription-related processes, endothelial cell proliferation regulation	2.1	.007
NM_008183	**Gstm2**	**Glutathione S-transferase mu 2**	**Transferase activity, glutathione metabolic process, xenobiotic process**	**2.0**	**.043**
NM_011171	Procr	Protein C receptor, endothelial	Immune response, signal transduction, blood coagulation	2.0	.034
NM_011480	Srebf1	Sterol regulatory element binding transcription factor 1	Transcription-related processes, heart rate regulation by chemical signal	2.0	.034
(b) Repressed by creosote at 22°C					
NM_022331	Herpud1	Homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1	Stress response, ER unfolded protein response, cellular Ca^{2+} homeostasis	3.3	.005
NM_025459	Fam134b	Family with sequence similarity 134 member B (mouse)	Pain sensory, stress response, transcription and DNA repair	3.3	.005
NM_001106610	Hdac11	Histone deacetylase 11	Cell differentiation, oligodendrocyte development, histone deacetylation	3.0	.002
NM_001034912	Fam134b	Family with sequence similarity 134 member B (rat)	Pain sensory, stress response, transcription and DNA repair	2.9	.002
NM_007468	Apoa4	Apolipoprotein A-IV	Innate immune response, lipid transport	2.7	.004
NM_001014058	Usp18	Ubiquitin-specific peptidase 18	Ubiquitin-dependent protein catabolic process	2.6	.012
NM_001126273	Alkbh2	alkB alkylation repair homolog 2, alpha-ketoglutarate-dependent dioxygenase	Transcription and DNA repair, oxidation–reduction reaction, pain sensory, stress response	2.6	.014
NM_177200	Svopl	SV2-related protein homolog (rat)-like	Transmembrane transport, establishment of localization	2.6	.008
NM_144549	Trib1	Tribbles homolog 1 (Drosophila)	Protein phosphorylation, regulation of protein kinase activity	2.5	.006
NM_001108341	Ulk1	Unc-51-like autophagy-activating kinase 1	Axonogenesis, Ras protein signal transduction, protein localization	2.5	.0002
NM_145076	Trim24	Tripartite motif-containing 24	Transcription and DNA repair, pain sensory, stress response	2.4	.009
NR_033304	Lypd6	LY6/PLAUR domain containing 6, transcript variant 2	Signalling, phosphorylation	2.3	.033
NM_028066	F11	Coagulation factor XI	Proteolysis, blood coagulation	2.3	.026
NM_177589	Ulk4	unc-51-like kinase 4	Protein phosphorylation, phosphate metabolic process	2.2	.013
NM_012050	Omd	Osteomodulin	Cell adhesion, extracellular region	2.2	.035
NM_008898	**Por**	**P450 (cytochrome) oxidoreductase**	**Regulation of monooxygenase activity, fatty acid oxidation**	**2.1**	**.004**
NM_053433	**Fmo3**	**Flavin-containing monooxygenase 3**	**Monooxygenase activity, oxidation–reduction process**	**2.1**	**.004**
NM_027498	Sik3	SIK family kinase 3	Protein phosphorylation	2.1	.0003
NM_028116	Pygo1	pygopus 1	Developmental process, Wnt receptor signalling pathway, kidney development	2.1	.007
NM_001011873	Xkr9	X Kell blood group precursor-related family member 9 homolog	Integral and intrinsic to membrane	2.0	.016

(Continues)

TABLE 5 (Continued)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
(c) Induced by creosote at 27°C					
NM_130408	Cyp26a1*	Cytochrome P450, family 26, subfamily a, polypeptide 1 (rat)	Retinoic acid catabolic and metabolic processes, central nervous system development	2.6	.017
NM_026871	Hint2	Histidine triad nucleotide binding protein 2	Hepatocellular carcinomas, steroid biosynthesis, apoptosis	2.5	.001
NM_007811	Cyp26a1*	Cytochrome P450, family 26, subfamily a, polypeptide 1 (mouse)	Retinoic acid catabolic and metabolic processes, central nervous system development	2.5	.024
NM_008061	G6pc	Glucose-6-phosphatase, catalytic	Gluconeogenesis, glycogen catabolic and metabolic processes	2.3	.011
NM_026301	Rnf125	Ring finger protein 125	Zinc ion binding, ligase activity	2.3	.025
(d) Repressed by creosote at 27°C					
NM_012543	Dbp	D-site of albumin promoter (albumin D-box) binding protein	Regulation of transcription (DNA dependent), regulation of cell proliferation, rhythmic process	5.4	.001
NM_017098	Fabp6	Fatty acid binding protein 6 ileal	Steroid and lipid metabolic processes	3.1	.020
NM_053716	Fbp2	Fructose-1,6-bisphosphatase 2	Gluconeogenesis, carbohydrate metabolic process	2.8	.042
NM_145572	Gys2	Glycogen synthase 2	Glycogen metabolic and biosynthetic processes, oxidation–reduction	2.2	.003
NM_013058	Id3	Inhibitor of DNA binding 3	Regulation of transcription and DNA replication	2.1	.049

Bolded transcripts are associated with detoxification of plant secondary compounds (*indicates transcripts induced at both temperatures). Associated functions compiled by GENESIFTER and supplemented from NCBI Biosystems.

4.2 | Response to dietary creosote varies with ambient temperature

Compound toxicity is exacerbated at warmer ambient temperatures for the desert woodrat, likely due to a decrease in overall liver function. By comparing the addition of creosote resin to a baseline control diet at two temperatures, we expected shifts in hepatic gene expression patterns at higher temperatures, specifically in genes associated with the biotransformation of xenobiotic compounds (Figure 1b, comparison B). More genes were differentially expressed at 22°C compared to 27°C (Table 2), as expected. Yet few detoxification-specific transcripts were differentially expressed at 22°C, no detoxification transcripts were differentially expressed at 27°C, and there were no xenobiotic-specific KEGG pathways listed as overrepresented at either 22°C or 27°C. In fact, more detoxification-specific genes appeared in comparisons testing the influence of temperature than in comparisons testing the effect of diet, outlined in the previous section. The lack of detoxification was surprising as plant secondary compounds such as alkaloids and terpenes induce a drug metabolism response in both laboratory and wild rodents (Magnanou et al., 2009; Settivari et al., 2006; Skopec, Haley, & Dearing, 2007). However, our results were in accordance with a more recent *Neotoma* study, which found that dietary toxins did not significantly impact the gene expression of xenobiotic metabolism pathways (Malenke et al., 2014). A similar trend was documented in a study investigating gene expression in insect herbivores, in that glucosinolates did not induce phase I or phase II detoxification genes as predicted but instead induced stress-related genes and pathways (Whiteman et al., 2012). It is possible that detoxification genes were constitutively expressed across all treatment groups. The differential gene expression of this study was only able to reveal changes in relative, not absolute, gene expression. Future studies utilizing transcriptomic approaches are necessary to fully address this hypothesis.

Only one gene was differentially expressed on a creosote diet at both temperatures, a cytochrome p450 from family 26 (Table 5). This gene, Cyp26a1, regulates and metabolizes retinoic acid, which is a metabolite of vitamin A and a known modulator of the retinoid X receptor (RXR; Ross & Zolfaghari, 2011). RXR in turn regulates the pathway of detoxification enzymes by binding with pregnane X receptor, thus creating a heterodimer that activates the transcription of mRNAs with biotransformation function, such as other cytochrome p450s and UDP glucuronosyltransferases (Tompkins & Wallace, 2007; Zhang, Xie, & Krasowski, 2008). Creosote could be influencing gene expression of Cyp26a1 in such a way that alters RXR and the regulatory pathway of detoxification, ultimately increasing the biotransformation of plant secondary compounds. Gene regulation is a crucial precursor to gene expression and ultimately enzymatic function, and therefore is a promising area of future work for elucidating the convoluted pathways of p450s as well as uncovering the mechanism of TDT.

TABLE 6 KEGG pathways overrepresented at 22°C (a, b) and at 27°C (c, d) due to dietary creosote (comparison B)

KEGG pathways	List	Gene set	*z*-score
(a) Pathways induced by creosote at 22°C			
Haematopoietic cell lineage	3	10	10.10
Regulation of actin cytoskeleton	2	54	2.33
Phagosome	2	47	2.59
Retinol metabolism	2	42	2.81
Osteoclast differentiation	2	28	3.67
Antigen processing and presentation	2	24	4.04
Amoebiasis	2	20	4.50
Cell adhesion molecules (CAMs)	2	16	5.12
Leishmaniasis	2	14	5.52
Intestinal immune network for IgA production	2	11	6.30
Type I diabetes mellitus	2	9	7.02
Graft-vs.-host disease	2	7	8.03
NOD-like receptor signalling pathway	1	17	2.28
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	1	16	2.37
Viral myocarditis	1	15	2.47
Hypertrophic cardiomyopathy (HCM)	1	13	2.71
Prion diseases	1	13	2.71
Dilated cardiomyopathy	1	11	3.00
Ether lipid metabolism	1	11	3.00
Primary immunodeficiency	1	7	3.89
ECM–receptor interaction	1	6	4.24
Allograft rejection	1	6	4.24
Asthma	1	6	4.24
Autoimmune thyroid disease	1	6	4.24
African trypanosomiasis	1	4	5.28
Malaria	1	3	6.15
(b) Pathways repressed by creosote 22°C			
Complement and coagulation cascades	1	36	2.86
mTOR signalling pathway	1	22	3.80
Regulation of autophagy	1	11	5.53
Fat digestion and absorption	1	10	5.82
(c) Pathways induced by creosote at 27°C			
Retinol metabolism	2	42	6.28
Insulin signalling pathway	1	51	2.65
Glycolysis/Gluconeogenesis	1	36	3.26
Starch and sucrose metabolism	1	23	4.20
Adipocytokine signalling pathway	1	21	4.41
RIG-I-like receptor signalling pathway	1	18	4.79
Carbohydrate digestion and absorption	1	10	6.54
Galactose metabolism	1	7	7.86
(d) Pathways repressed by creosote at 27°C			
Insulin signalling pathway	2	51	5.65
PPAR signalling pathway	1	37	3.21
Glycolysis/Gluconeogenesis	1	36	3.26
Starch and sucrose metabolism	1	23	4.20
TGF-beta signalling pathway	1	21	4.41
Fructose and mannose metabolism	1	17	4.94
Pentose phosphate pathway	1	10	6.54

"List" indicates the number of genes highly expressed within the pathway. "Gene set" indicates the total number of genes in that pathway included on the array.

Woodrats responded differently to dietary creosote based on ambient temperature. There was no overlap in the genes differentially expressed by animals feeding on creosote housed at 22°C and 27°C (Table 5), except for Cyp26a1 as discussed above. The lack of similarity across these transcript lists suggests that woodrats may be mounting diverse responses to creosote at different ambient temperatures. The interaction between temperature and dietary toxins could have also influenced this pattern in gene expression, which is discussed in greater detail later. Additionally, creosote had a greater effect on differential gene expression at 22°C compared to 27°C. More transcripts were differentially expressed at 22°C compared to 27°C (Table 5), implying that there was more flexibility in the liver at a cooler temperature compared to a thermoneutral temperature. Furthermore, the clustering of individual woodrats based on overall hepatic gene expression suggested that, regardless of diet treatment, animals at 27°C behaved in a similar manner as all individuals at that temperature clustered together (Figure 2). Conversely, woodrats at the cooler temperature clustered separately based on diet treatments. All animals ingesting creosote resin at 22°C formed a unique cluster, indicating that these woodrats were most similar to themselves with a novel expression profile compared to woodrats ingesting a control diet (Figure 2).

The disparate profiles of creosote-mediated gene expression across ambient temperatures may be an example of temperature constraints that challenge herbivorous mammals. Animals could be in an energy-limited state at warmer temperatures, as genes and KEGG pathways associated with gluconeogenesis were significantly influenced by creosote at 27°C (Tables 5 and 6). Other work has documented gluconeogenesis as a significantly upregulated pathway in laboratory rats given dietary toxins and housed at higher temperature (Settivari et al., 2006, 2009). Likewise, the expression of a mitochondrial energy synthesis gene was significantly upregulated in liver of Japanese quail (adenine nucleotide translocator (ANT), Voltolini et al., 2014). Conversely, cooler temperatures could represent a "release state" in the liver whereby more gene expression is permitted by lower temperatures. Almost fivefold more genes were differentially expressed due to ingestion of dietary creosote at 22°C compared to 27°C (Table 5). In addition, half of the overrepresented KEGG pathways induced by creosote at 22°C were linked to lysosome and intraorganelle membrane function (RT1-Da), which could be a sign of high cellular turnover and overall growth (Lee, Giordano,

TABLE 7 Transcripts differentially expressed at 27 and 22°C while woodrats were ingesting diets with differing concentrations of creosote resin (7% and 4%, respectively)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
(a) Upregulated at 27°C					
NM_010009	Cyp27b1	Cytochrome P450 family 27 subfamily b polypeptide 1	Steroid metabolic process, calcium ion transport, ageing, regulation of cell proliferation	3.1	.036
NM_008380	Inhba	Inhibin beta-A	Apoptosis induction, cell cycle arrest	2.8	.019
NM_001161712	**Gcat**†	**Glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase) transcript variant 2**	**Peptide GPCRs, metabolism of glycine, serine and threonine**	**2.3**	**.015**
NM_027430	Mpc2	Mitochondrial pyruvate carrier 2	Glycolysis, gluconeogenesis	2.3	.003
NM_016668	**Bhmt**†	**Betaine-homocysteine methyltransferase**	**Protein methylation, carboxylic acid metabolic process**	**2.3**	**.004**
NM_007820	**Cyp3a16***	**Cytochrome P450 family 3 subfamily a polypeptide 16**	**Oxidation–reduction process, monooxygenase activity**	**2.2**	**.012**
NM_019879	Suclg1	Succinate-CoA ligase GDP-forming alpha subunit	Tricarboxylic acid cycle, succinate metabolic process, oxidation–reduction process	2.2	.012
NM_028066	F11	Coagulation factor XI	Proteolysis, blood coagulation	2.2	.001
NM_020565	**Sult3a1**†	**Sulfotransferase family 3A member 1**	**Sulfotransferase activity (amine), transferring sulphur-containing groups, catalytic activity**	**2.1**	**.005**
NM_020565	**Sult3a1**†	**Sulfotransferase family 3A member 1**	**Sulfotransferase activity (amine), transferring sulphur-containing groups, catalytic activity**	**2.1**	**.004**
NM_012520	**Cat***	**Catalase**	**Response to reactive oxygen species and to oxidative stress**	**2.1**	**.001**
NM_001130490	Lrp1	Low-density lipoprotein-related protein 1	Metabolism (fat-soluble vitamins, vitamins and cofactors, retinoid)	2.1	.005
NM_007410	**Adh5***	**Alcohol dehydrogenase 5 (class III) chi polypeptide**	**Retinoid metabolic process, respiratory system process, ethanol oxidation**	**2.1**	**.015**
NM_001039219	Gm6086	Predicted gene 6086	Transferase and catalytic activity	2.0	.017
NM_025291	Sra1	Steroid receptor RNA activator 1 transcript variant 1	Transcription (DNA dependent), regulation of transcription	2.0	.001
(b) Upregulated at 22°C					
NM_026672	**Gstm7**†	**Glutathione S-transferase mu 7**	**Glutathione transferase activity, transferring alkyl or aryl (other than methyl) groups**	**6.2**	**.032**
NM_026672	**Gstm7**†	**Glutathione S-transferase mu 7**	**Glutathione transferase activity, transferring alkyl or aryl (other than methyl) groups**	**5.3**	**.032**
NM_001008847	RT1-Da	RT1 class II locus Da mRNA	Lysosome, lysosome membrane, regulation of enzymatic activity	4.6	.016
NM_153468	Gzma	Granzyme A	Proteolysis, regulation of endodeoxyribonuclease activity	4.5	.009
NM_008183	**Gstm2**†	**Glutathione S-transferase mu 2**	**Glutathione metabolic process, xenobiotic catabolic process**	**3.1**	**.039**
NM_013470	Anxa3	Annexin A3	Hepatocellular carcinomas, cell migration, phagocytosis	2.8	.039
NM_019291	Car2	Carbonic anhydrase II	Kidney development, one-carbon metabolic process, response to stress	2.5	.004
NM_178847	Cyp27a1	Cytochrome P450 family 27 subfamily a polypeptide 1	Catabolic processes (cholesterol, steroid), oxidation–reduction process	2.3	.038

(Continues)

TABLE 7 (Continued)

Accession no.	Gene ID	Gene description	Associated function	Fold change	*p*-value
NM_011921	**Aldh1a7***	**Aldehyde dehydrogenase family 1 subfamily A7**	**Retinoic acid metabolic process, protein homotetramerization**	**2.3**	**.018**
NM_172759	**Ces2e***	**Carboxylesterase 2E transcript variant 1**	**Hydrolase activity (acting on ester bonds), carboxylesterase activity**	**2.2**	**.001**
NM_172759	**Ces2e***	**Carboxylesterase 2E transcript variant 1**	**Hydrolase activity (acting on ester bonds), carboxylesterase activity**	**2.2**	**.001**
NM_013467	**Aldh1a1***	**Aldehyde dehydrogenase family 1 subfamily A1**	**Response to oxidative stress, response to drug, retinol metabolic process**	**2.1**	**.010**
NM_001190330	**Ces2a***	**Carboxylesterase 2A transcript variant 2**	**Hydrolase activity, biological oxidations, metabolism**	**2.1**	**.007**
NM_053262	Hsd17b11	Hydroxysteroid (17-beta) dehydrogenase 11	Oestrogen biosynthesis, metabolism (steroid hormone, lipid, lipoprotein)	2.1	.005
NM_001191676	**Ugt2b10†**	**UDP glucuronosyltransferase 2 family polypeptide B10**	**Glucuronate pathway, ascorbate biosynthesis, glucuronidation (flavonoid, cellular), metabolism**	**2.1**	**.008**
NM_001007602	**Gsto1†**	**Glutathione S-transferase omega 1**	**L-ascorbic acid biosynthetic process, carboxylic acid metabolic process**	**2.1**	**.025**
NM_011921	**Aldh1a7***	**Aldehyde dehydrogenase family 1 subfamily A7**	**Retinoic acid metabolic process, protein homotetramerization**	**2.1**	**.025**
NM_001013084	**Akr1b10***	**Aldo-keto reductase family 1 member B10 (aldose reductase)**	**Catalytic and oxidoreductase activity, oxidation–reduction process**	**2.1**	**.008**
NM_001007699	Cept1	Choline/ethanolamine phosphotransferase 1	Phospholipid biosynthetic process, organophosphate metabolic process	2.1	.001
NM_001191676	**Ugt2b10†**	**UDP glucuronosyltransferase 2 family polypeptide B10**	**Glucuronate pathway, ascorbate biosynthesis, glucuronidation (flavonoid, cellular), metabolism**	**2.0**	**.008**

Bolded transcripts indicate function related to detoxification or xenobiotic metabolism (*indicates phase I detoxification; †indicates phase II detoxification). Associated functions compiled by GENESIFTER and supplemented from NCBI Biosystems.

& Zhang, 2012; McBride & Kelly, 1990). Lastly, 15 of 26 overrepresented KEGG pathways were associated with human disease (Table 6), which could indicate that the liver has the flexibility to turn on numerous genes and pathways at 22°C.

4.3 | Potential interactions between ambient temperature and dietary toxins

Although animals on the creosote diet treatment were ingesting unequal amounts of creosote resin, which could impact detoxification mechanisms (e.g., Ioannides, 1999; Roos, Tschirbs, Hack, Welge, & Wilhelm, 2004; Skopec et al., 2007), patterns in differential gene expression were apparent (Figure 1b, comparison C). Animals at the thermoneutral temperature ingested 50% more creosote resin than woodrats at the cooler temperature, yet more transcripts were differentially expressed at 22°C compared to 27°C, including a high number of detoxification-specific transcripts (Table 7). This result is similar to our other comparisons in this study, in that more transcripts were expressed at the cooler temperature. In addition, some of the genes differentially expressed at 22°C were previously identified by Malenke et al. (2014) to be induced by creosote resin. Moreover, the fold changes for transcripts of animals ingesting creosote resin at 22°C were the highest values recorded in our analysis, with a glutathione S-transferase mu 7 displaying a 6.15× fold change (Table 7). The increased expression of these genes could be indicative of substantial detoxification. This outcome further supports the idea that cooler temperatures do not restrict gene expression in the liver. Lastly, despite animals at 27°C facing a larger toxic challenge, overall gene expression in woodrats at the thermoneutral temperature did not differ greatly based on diet, whereas the ingestion of creosote resin caused the animals at 22°C to have more similar gene expression profiles (Figure 2). Overall, this pattern provides additional evidence that fewer genes may be turned on, resulting in fewer changes in differential gene expression, at warmer temperatures compared to cooler temperatures.

Detoxification genes from disparate enzyme families were differentially expressed at 22°C compared to 27°C in woodrats ingesting creosote diets. For instance, glutathione S-transferases, aldehyde dehydrogenases and carboxylesterases were upregulated at 22°C, while various transferases were upregulated at 27°C, including sulfotransferases; however, UGTs were notably absent (Table 7). While purely speculative, it is possible that sulfotransferases were upregulated at 27°C because UGTs were preferentially utilized at warmer temperatures to regulate steroid biosynthesis, as discussed above.

Regardless of the caveats associated with this comparison, our results shed light on effects that ambient temperature can have on hepatic gene expression in woodrats.

4.4 | Beyond herbivorous rodents

Current literature on temperature-mediated changes in gene expression mainly encompasses ectotherms due to their more-or-less direct behavioural and physiological response to heat (i.e., Bale et al., 2002). However, the patterns we report here for woodrats, an endothermic mammal, are remarkably similar to previous work in fish. A pivotal paper by Podrabsky and Somero (2004) stated that constant vs. fluctuating temperatures alter gene expression in fish. Cell growth and proliferation controls were constant in response to temperature, but other pathways became important in conditions of controlled or fluctuating temperatures, such as carbohydrate metabolism and membrane structure maintenance (Podrabsky & Somero, 2004). Furthermore, a chemical toxicology study in fish found that cadmium toxicity increased at higher temperatures (Vergauwen, Hagenaars, Blust, & Knapen, 2013). The underlying mechanism was likely a change in toxin sensitivity via accumulation of cadmium in the tissue rather than direct liver oxidative damage (Vergauwen et al., 2013). However, the group also found that gluconeogenesis genes were reliable markers of cold-induced oxidative stress (Vergauwen et al., 2013). These studies highlight the potential for conserved and widespread negative responses to temperature and toxins across unrelated taxa regardless of thermoregulatory strategy.

The work presented in this study supported the prediction that fewer genes would be expressed at higher ambient temperatures, but also lent to new speculation about the mechanism of TDT. Changes in blood flow could be partially responsible for TDT, because blood flow is known to be diverted to the periphery and away from the viscera as a thermoregulatory response in mammals at warmer temperatures (Dearing, 2013; Grayson, Irvine, & Kinnear, 1966). However, blood shunting is an immediate, physiological response to thermal stress that is often short term and potentially energetically expensive (Hales et al., 1979; Reichard, Prajapati, Austad, Keller, & Kunz, 2010). It is probable that multiple pathways pertaining to thermoregulation and detoxification are acting in concert, ultimately resulting in TDT. Future avenues of research could include measuring liver damage with conventional assays, such as serum levels of alanine transaminase (Kim, Flamm, Di Bisceglie, & Bodenheimer, 2008; Ozer, Ratner, Shaw, Bailey, & Schomaker, 2008), to better define hepatic stress. Additionally, monitoring core body temperature across temperature and dietary treatments could provide more information regarding the environment in which the liver is functioning (Rothwell & Stock, 1980; Settivari et al., 2006).

Lastly, our results should be considered within the context of global climate change due to the implications of TDT and potential effects on ecologically and evolutionarily relevant systems. While animals from diverse environments have different ranges of thermal tolerance and thus varying abilities to adapt to climate change (Tomanek, 2008), mammalian herbivores of all sizes will likely face the combined challenge of dietary toxins and rising ambient temperature. In addition, the historically unprecedented changes in climate will undoubtedly play an important role in how mammalian herbivores will respond to TDT (Field et al., 2014), which in turn could affect their foraging behaviour and ability to survive in a warming climate. Thus, understanding the mechanistic nature of temperature-dependent toxicity will not only advance the field of herbivore ecology but also inform predictions about mammalian species' responses to environmental changes in their natural environment.

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DATA ACCESSIBILITY

All raw microarray data: Gene Expression Omnibus, NCBI, series record GSE80595. Feeding trial data (food intake, body mass, etc.) and analysis (R script): Dryad https://doi.org/10.5061/dryad.230v7.

AUTHOR CONTRIBUTIONS

M.D.D., P.K.C. and J.R.M. conceived and designed the research. P.K.C. conducted experiments and collected data. P.K.C. and J.R.M. analysed data. All authors interpreted the findings. P.K.C. wrote the manuscript. All authors contributed substantially to article revisions and approved the content of the final manuscript.

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SUPPORTING INFORMATION

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