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Hepatic gene expression in herbivores on diets with natural and novel plant secondary compounds

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Magnanou E, Malenke JR, Dearing MD. Hepatic gene expression in herbivores on diets with natural and novel plant secondary compounds. *Physiol Genomics* 45: 774–785, 2013. First published July 16, 2013; doi:10.1152/physiolgenomics.00033.2013.—Herbivores are predicted to evolve appropriate mechanisms to process the plant secondary compounds (PSCs) in their diet, and these mechanisms are likely specific to particular suites of PSCs. Changes in diet composition over evolutionary time should select for appropriate alterations in metabolism of the more recent dietary components. We investigated differences in gene expression profiles in the liver with respect to prior ecological and evolutionary experience with PSCs in the desert woodrat, *Neotoma lepida.* This woodrat species has populations in the Mojave Desert that have switched from feeding on juniper to feeding on creosote at the end of the Holocene as well as populations in the Great Basin Desert that still feed on the ancestral diet of juniper and are naïve to creosote. Juniper and creosote have notable differences in secondary chemistry. Woodrats from the Mojave and Great Basin Deserts were subjected to a fully crossed feeding trial on diets of juniper and creosote after which their livers were analyzed for gene expression. Hybridization of hepatic mRNAs to laboratory rat microarrays resulted in a total of 20,031 genes that met quality control standards. We analyzed differences in large-scale patterns of liver gene expression with respect to GO term enrichment. Diet had a larger effect on gene expression than population membership. However, woodrats with no prior evolutionary experience to the diet upregulated a greater proportion of genes indicative of physiological stress compared with those on their natural diet. This pattern may be the result of a naïve animal's attempting to mitigate physiological damage caused by novel PSCs.

biotransformation; gene expression; PSCs; liver; microarray; woodrat

PLANT-HERBIVORE INTERACTIONS are crucial interspecies relationships, and the coevolutionary processes underlying these interactions have been the center of many studies (Refs. 2, 14, 15, 25 for review and examples). Recent advances in molecular approaches facilitate the characterization of biochemical processes associated the interactions between plants and their herbivores (3). For example, studies of gene expression profiles of herbivores in response to plant secondary compound (PSC) ingestion have revealed that the herbivore's response is dependent on the chemical class of the PSC consumed (4, 13, 24). Transcriptomic approaches have demonstrated the profound physiological adjustments that occur during radical changes in diet (5, 30).

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Cases in which herbivores undergo dietary shifts across classes of PSCs represent opportunities to identify candidate genes that may have been favored to process a given set of dietary toxins. The desert woodrat, *Neotoma lepida,* is a unique mammalian model for studying a switch from one type of toxic diet to another. During the establishment of the Mojave Desert in the Holocene (~17,000 yr ago), woodrats occupying the southwestern USA underwent a major shift from feeding on a diet of juniper, *Juniperus spp*., to that of a natural invader, creosote, *Larrea tridentata* (16, 20, 38). Currently, creosote can constitute up to 75% of the diet of woodrats in the Mojave (20). *Larrea tridentata* and *Juniperus spp*. have different PSC profiles. Juniper contains numerous terpenes (>35 monoterpenes) that can constitute up to 5% of the dry weight (dw) as well as less abundant tannins (1, 31, 34). In contrast, creosote leaves are coated with a complex resin composed of numerous polyphenolic compounds (26). Resin content of the leaves can vary from 10–25% (dw), and its primary component is nordihydroguaiaretic acid (NDGA). Thus, the switch from a diet of juniper to that of creosote presents new metabolic challenges for woodrats. In the Great Basin, where creosote is not present, populations of desert woodrats still feed on juniper (*J. osteosperma*). These woodrats have no evolutionary or ecological experience with creosote and diverged from the Mojave Desert populations only within the past 60,000 yr (32). The Great Basin woodrats have a lower tolerance to *L. tridentata* compared with woodrats from the Mojave (28, 29). Thus, the historic diet switch of desert woodrats from juniper to creosote coupled with extant populations that feed on the ancestral diet permits a comparison that may enable the identification of metabolic pathways with respect to dietary PSCs.

Previously, in a report on the impact of juniper and creosote PSCs on gene expression of hepatic biotransformation (aka detoxification) enzymes in both Mojave and great Basin populations of *N. lepida* we used microarrays designed for *Rattus norvegicus* (27). We found that woodrats employ distinct biotransformation pathways to metabolize creosote and juniper. In addition, Mojave woodrats utilize a unique set of biotransformation enzymes for metabolizing creosote compared with conspecifics from Great Basin Desert that were naïve to creosote. This study shed light on the physiological adjustments of the detoxification system of woodrats to a novel set of toxins.

The goal of this new research was to expand from the previous work focused on biotransformation processes to more comprehensive metabolic processes. We investigated hepatic gene expression for >32,000 unique genes of Mojave and Great Basin woodrats fed juniper and creosote diets (27) to develop an overview of differential gene expression of animals

on diets with different PSC classes. Herbivores that consistently feed on particular PSCs are thought to have evolved efficient biotransformation strategies for those PSCs (9, 22). Biotransformation is the enzymatic conversion of xenobiotics. It produces more hydrophilic metabolites of PSCs that may or may not be less toxic than the parent compound. We hypothesized that populations with previous long-term experience to a class of PSCs would upregulate fewer overall genes when fed these PSCs than when exposed to novel one. Moreover, we expected that herbivores with no prior experience to a diet with novel PSCs would be less likely to efficiently biotransform these compounds and present a pattern of gene expression more indicative of metabolic stress than populations with previous experience.

MATERIALS AND METHODS

Woodrat trapping and feeding trials. Wild-caught woodrats were used for all experiments. All experimental procedures were approved by the University of Utah's Institutional Animal Care and Use Committee (protocol number 07-02015). We live-trapped 13 (7 females, 6 males) and 17 (5 females, 12 males) *N. lepida,* respectively, from the same genetic subclade in two distinct habitats: the Great Basin Desert (Tooele, UT) and the Mojave Desert (Washington, UT). All the woodrats were transported to the University of Utah Department of Biology's Animal Facility. They were housed in individual cages and were acclimated to captivity for 3 mo before the experiment. Woodrats were fed standard rabbit chow (Harlan Teklad formula 2031); water was provided ad libitum.

We designed a fully crossed, two-by-two feeding trial from these two populations fed diets of juniper and creosote bush (Fig. 1*A*). Four Great Basin and four Mojave woodrats were fed a rabbit chow diet supplemented with juniper foliage (2 females and 2 males of each). An additional four animals from each population (2 females, 2 males from the Great Basin and 4 males from the Mojave) were fed a diet supplemented with resin extracted from the foliage of the creosote bush. Foliage was collected from the respective desert habitats and stored at −20°C until use. Diets differed primarily in PSC content.

Experimental animals were fed a gradually increasing amount of juniper or creosote resin over a 5-day period to allow induction of biotransformation pathways (see Ref. 27 for details on diet preparation and PSC concentrations). Animal mass and the dry matter intake per animal were recorded daily.

Microarray experiment. After the feeding trial, animals were euthanized. Livers were removed, weighed, cut into subsamples (~20 mg), and individually incubated overnight at 4°C in RNA Later (Ambion). The liver samples were removed from the solution and stored at −80°C.

Total RNA from the liver sections was extracted with Tri Reagent (Sigma) per the manufacturer's protocol. The samples were purified with a DNase treatment from RNaqueous-4PCR (Ambion). The quality of the RNA was assessed with an RNA Monochip Bioanalyzer system (Agilent Technologies), and quantity was determined by a Nanodrop ND-1000 spectrophotometer. Total RNA (500 ng/sample) was labeled with Cyanine-3 CTP or Cyanine-5 CTP using an Agilent Low RNA Input Linear Amplification kit as specified by the manufacturer. Gene expression hybridizations were performed with the Agilent Gene Expression Hybridization kit following the manufacturer's instructions.

The fluorescently labeled amplified RNA were hybridized to Agilent Technologies 60 mer oligonucleotide rat (*R. norvegicus*) microarrays (G4131F) per the manufacturer's instructions. More than 41,000 rat genes and transcripts were represented on the arrays. A total of 16 arrays, one per individual woodrat, were used in a reference design (Fig. 1*B*). A common reference of total RNA pooled from an equal amount of the 16 samples was used. Following hybridization, the gene

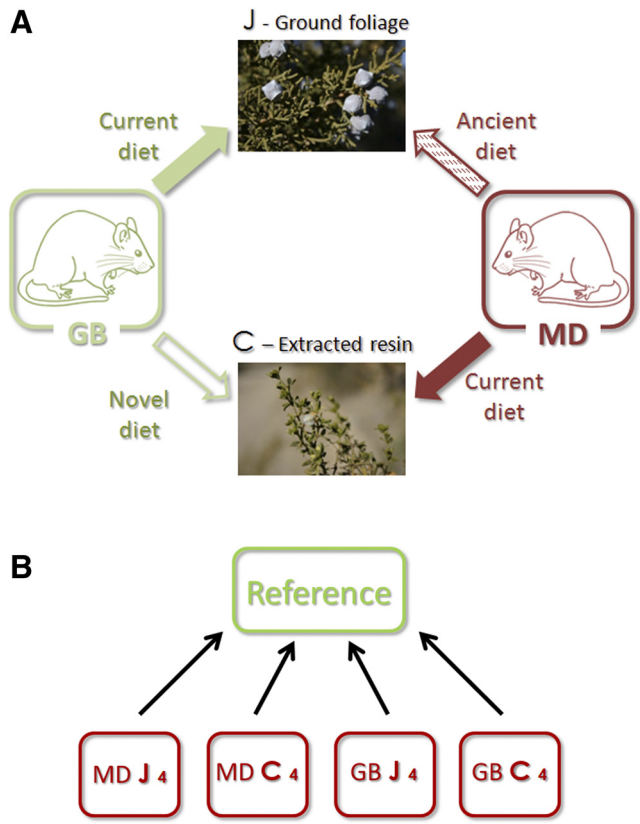

Fig. 1. Feeding trial and microarray experimental designs. *A*: feeding trial experimental design. A fully crossed, 2×2 feeding trial was used with the 2 populations of the desert woodrat and diets of juniper and creosote bush. Juniper is presumed to be the ancestral diet of the Mojave woodrats and is currently included in the diets of Great Basin woodrats. In contrast, creosote only occurs in the diets of Mojave woodrats and is novel to Great Basin animals. *B*: microarray experimental design using a reference RNA sample. The reference RNA sample consisted in the equimolar pool of the 16 RNAs coming from the 4 treatments. Boxes, representing mRNA samples, are labeled as treatments. Subscripts indicate the number of independent biological replicates (1 woodrat per array) of the same treatment. Arrows represent hybridizations between the mRNA samples and the microarray. The sample at the tail of the arrow is labeled with red (Cy5) dye, and the sample at the head of the arrow is labeled with green (Cy3) dye. MD, Mojave Desert woodrat population; GB, Great Basin woodrat population; J, juniper diet; C, creosote diet.

expression microarrays were separated from the gasket slide and washed according to the manufacturer's protocol. The stringent wash step was performed at room temperature to optimize this procedure for a heterologous hybridization (woodrat on a rat array). Microarrays were scanned on an Agilent Technologies G2565BA Microarray Scanner System.

We used Feature Extraction 9.1.3.1 software (Agilent Technologies) to determine feature intensities and ratios, reject outliers, as well as normalize dye data (linear lowess), and to generate quality control reports. Data were exported to txt-format files. Normalized ratios were $\log_2$ transformed before being analyzed with GeneSpring GX11 (Agilent Technologies). Data have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) (7) and are accessible through GEO Series accession number GSE44411. The following link allows review of record GSE44411 while it remains in private status: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vvurraeaekqcwnw&acc=GSE44411.

Quantitative real-time PCR were conducted on three biotransformation genes and validated the accuracy of microarray for gene expression characterization: superoxide dismutase, cytochrome P450 2A3A, and P450 (cytochrome) oxidoreductase (27).

Data analysis. We limited our analysis in this study to the 39,526 probes on the array that corresponded to 32,818 different genes for which a function is currently predicted or known. To determine the patterns of variation in gene expression between treatments, we conducted a principal component analysis (PCA, GeneSpring); only ratios of genes with good quality signal (i.e., intensities >1.00) for all individuals were included in the PCA. We probed the differences between population and diet treatments with paired comparisons. Individual transcripts were included when at least three individuals per population had signal intensity >1.00. Transcripts were plotted adapting the vector analysis from (19). They were sorted according to their pattern of expression (fold change, *P* value). Sets of differentially expressed genes (paired *t*-test, $P < 0.05$) were screened for Gene Ontology (GO) terms enrichment using GeneSpring GX11.5 at all levels of the ontology tree and for the three categories (biological processes, molecular function, cellular components; $P < 0.01$ applying a Bonferroni type I error correction). Some specific comparisons required a focus on *level 2* of biological processes terms. We compared gene expression between diets for each population independently to determine whether dietary differences caused large differences in liver expression patterns. We addressed the question of the contribution of biotransformation processes assessing either the effect of diet or population on gene expression applying the same statistics to biotransformation genes and to unique known gene lists with no

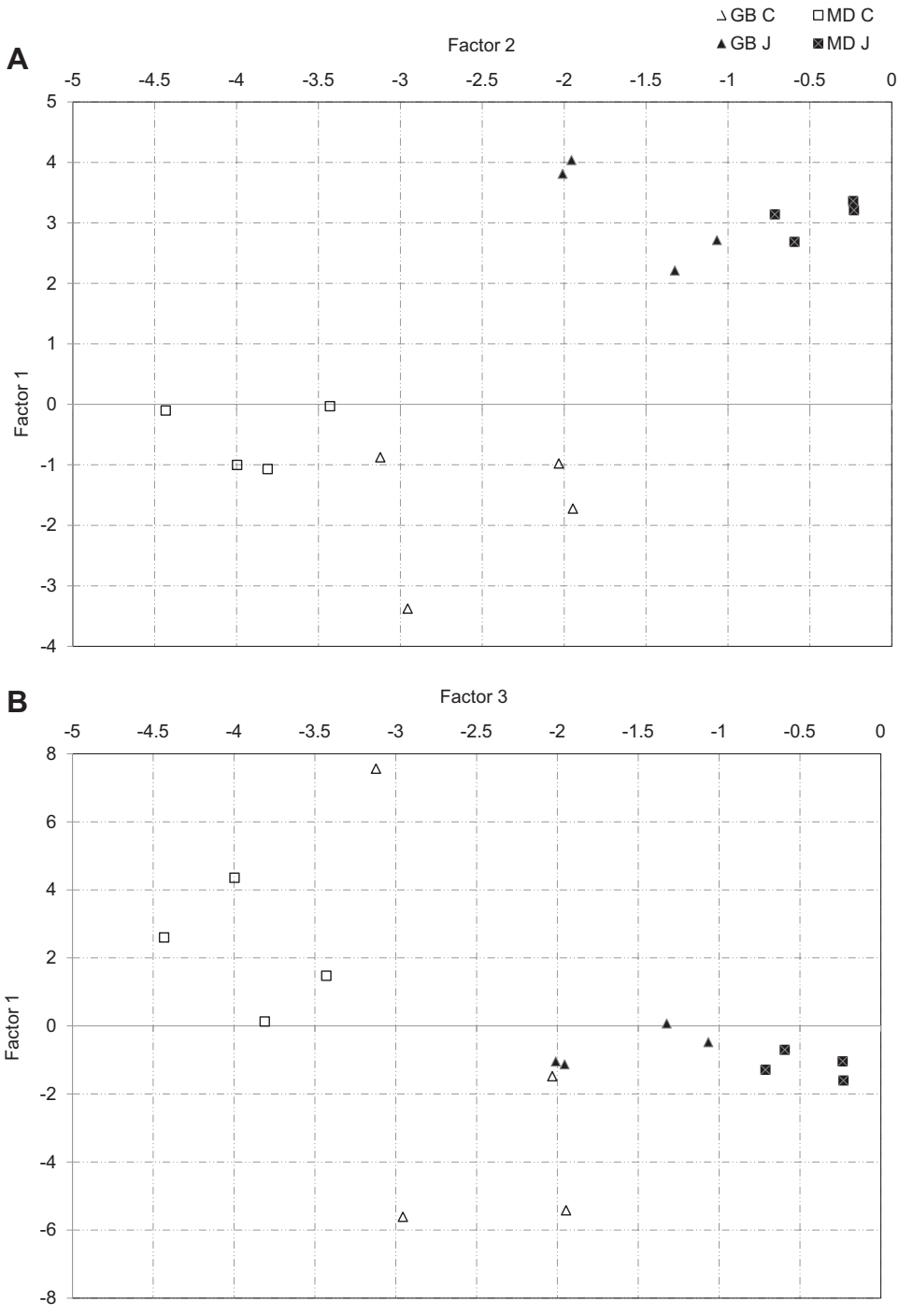

Fig. 2. The expression pattern of annotated transcripts of 16 *Neotoma lepida* is represented as a function of the 3 first factors obtained in a principal component analysis (PCA). PCA was based 20,031 genes with an intensity of 1 for all 16 woodrats. *Factors 1, 2,* and *3* accounted for 51% of the total variance. Triangles, GB woodrats; squares, MD woodrats. solid symbols, J diet; open symbols, C diet. *A*: *factors 1* and *2* best represented the overall data variation, i.e., both diet and population membership shaped gene expression. *B*: *factors 1* and *3* allowed observing a significantly higher dispersion of individuals when fed creosote rather than when fed juniper.

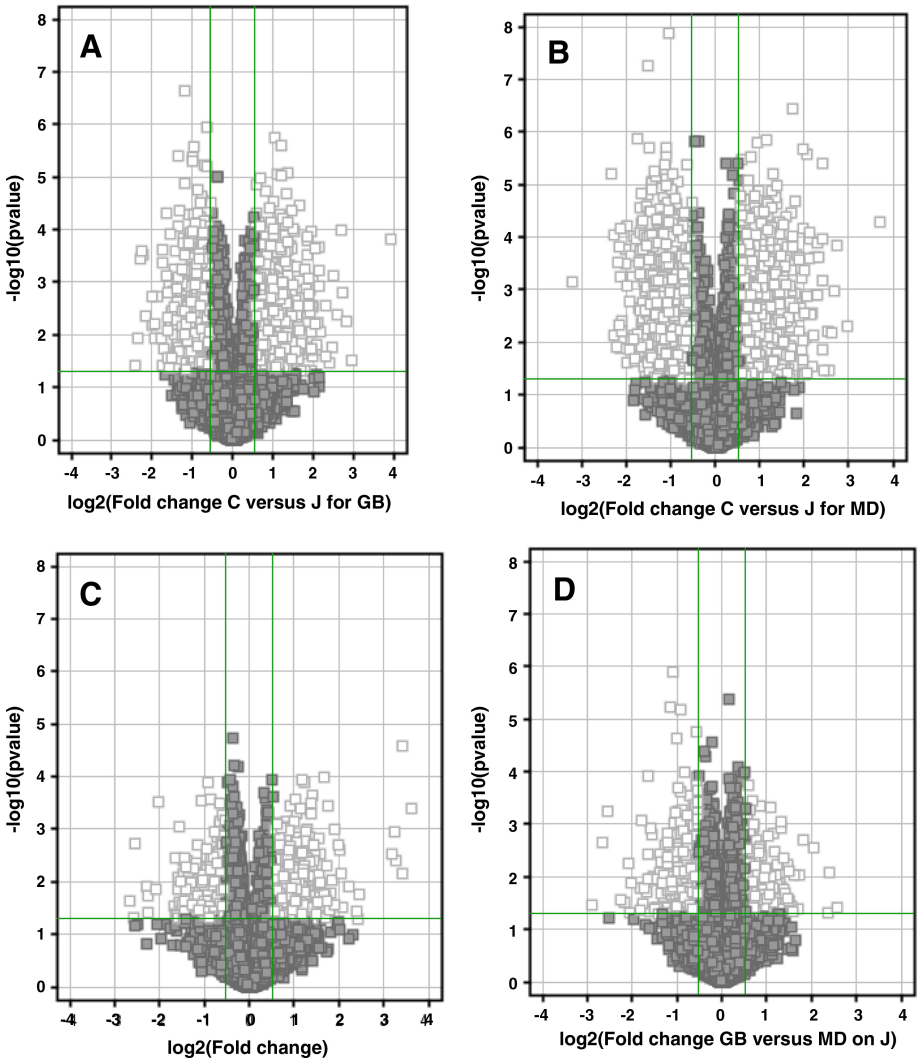

Fig. 3. Volcano plots of significance vs. fold change in hepatic expression of genes in the 4 comparisons investigated. Each square represents analysis (paired *t*-test) of expression for a single gene. The white squares represent genes with larger fold differences (fold change >1.5) and higher significance values ($P < 0.05$). Expression difference is plotted between GB woodrats on J vs. C (*A*), MD woodrats on J vs. C (*B*), GB vs. MD animals on the C diet (*C*), GB vs. MD on the J diet (*D*).

redundant probes (paired *t*-test, raw $p < 0.05$, fold change >1.5) to make the comparison similar.

The results from microarrays can be analyzed by a variety of statistical techniques ranging from conservative to liberal with respect to the number of genes identified as significant [e.g., Storey's Q, $P < 0.001$ (36)]. Because the fold change in expression is more consistent across laboratories and platforms (11), we adopted the procedure suggested by MicroArray Quality Control project to rank order genes

Table 1. *Number of transcripts that passed the quality control and total number of significant changes in gene expression for each pair of comparisons: effect of diet on a given population*

	Mojave	%	Great Basin	%
Adequate quality transcripts				
Total	25,831	79	25,697	78
Significantly expressed transcripts				
Total	3,109	12	1,751	7
Upregulated on J	1,834	**59**	735	42
Upregulated on C	1,275	41	1,016	**58**

Significant changes were characterized as regulated either up or down. Columns indicate the percentage of transcripts that passed the quality control per the number of elements on the array, and the percentage of differentially expressed genes per the total number of transcripts involved in the *t*-test (raw $P < 0.05$). The percentages of overexpressed genes for a given population on the novel diet are indicated in boldface. J, juniper diet; C, creosote diet.

Table 2. *Number of transcripts that passed the quality control and total number of significant changes in gene expression for each pair of comparisons: population effect for a given diet*

	C	%	J	%
Adequate quality transcripts				
Total	24,781	76	27,511	84
Significantly expressed transcripts				
Total	1,099	4	749	3
Upregulated for MD	397	36	481	**66**
Upregulated for GB	702	**64**	268	**34**

Significant changes were characterized as regulated either up or down. Columns indicate the percentage of transcripts that passed the quality control per the number of elements on the array, and the percentage of differentially expressed genes per the total number of transcripts involved in the *t*-test (raw $P < 0.05$). The percentages of overexpressed genes for a given diet on the novel diet are indicated in boldface. MD, Mojave Desert population; GB, Great Basin population.

significant at the $P < 0.05$ level by fold-change levels >1.5 (11, 21). While this statistical analysis will have a greater false discovery rate than the Storey's Q, it is more appropriate for gene discovery especially in a cross-species study.

RESULTS

Feeding trials. There was no difference in body mass between the four experimental groups (136.82 ± 5.48 g; ANOVA $F_{3,12} = 0.319$, $P = 0.811$) before the beginning of the experiments. Both populations responded similarly to the juniper diet, consuming similar amounts of juniper and ending the trial in equivalent negative mass balance. However, on the creosote bush diet, Mojave Desert woodrats consumed more creosote resin and maintained positive mass balance, whereas Great Basin Desert woodrats consumed ~30% less resin and ended in negative mass balance (27).

Overall pattern of gene expression. Of the 32,818 unique genes with known function on the array, 72% had an average quality control index per treatment of 0.75, i.e., three of four individuals had quality control index of 1. Of the 32,818 elements on the array, 20,031 exhibited adequate quality for incorporation into the PCA (i.e., all 16 individuals met the quality threshold of 1). There was a strong effect of diet on gene expression in both populations (GeneSpring GX11; Fig. 2, *A* and *B*). The first seven factors of the PCA accounted for 83% of the total variance, while the first three factors accounted for 51%. No single gene contributed significantly to these factors. Overall, both population membership and diet affected gene expression patterns (Fig. 2*A*). There was also a significant difference in the intragroup standard deviation of the four treatments with respect to *factor 1* and *3* coordinates (Bartlett test $P < 0.5$ and 0.001, respectively). Gene expression was more variable on the creosote diet than the juniper

Table 3. *Differentially expressed genes that exhibit the same change in expression between the two populations of GB and MD woodrats regardless of diet*

Gene ID	Gene Name	Log_2 ratio on C	Log_2 ratio on J
	Greater expression for GB		
NM_001006993	regulator of calcineurin 2 (Rcan2)	12.15	5.27
AW140877	BI289132 UI-R-DK0-cfe-h-02-0-UI.s1 UI-R-DK0 Rattus norvegicus cDNA clone UI-R-DK0-cfe-h-02-0-UI 3¢, mRNA sequence [BI289132]	9.66	5.09
BI289132	sarcoglycan, gamma (dystrophin-associated glycoprotein)	9.38	5.94
XM_001076936	PREDICTED: hypothetical protein LOC691130	3.40	3.24
XM_233609	Q6TUG9_RAT (Q6TUG9) LRRGT00075, partial (10%) [TC611781] similar to *H. sapiens* mRNA sequence AW140877	2.86 8.97	3.00 2.91
NM_147177	ribosomal protein L24	2.22	2.80
NM_207597	RuvB-like 1 (*E. coli*)	2.83	2.77
NM_022515	olfactory receptor 1078	2.31	2.31
AI010960	interleukin 13 receptor, alpha 1 (Il13ra1)	1.63	2.18
XM_001072122	BG665185 DRABZF03 Rat DRG Library *Rattus norvegicus* cDNA clone DRABZF03 5¢	1.69	2.10
NM_001115027	Ubiquitin-specific peptidase 49 (Usp49)	1.50	1.84
XM_341701	Rap1 gap protein Fragment	2.98	1.79
NM_175578	ATP-binding cassette, subfamily G (WHITE), member 5 (Abcg5)	1.81	1.79
NM_139110	G protein-coupled receptor 116 (Gpr116)	1.86	1.77
NM_001126080	EST205411 from Normalized rat muscle	2.10	1.75
NM_022599	similar to RIKEN cDNA 2310007F12	2.06	1.71
NM_053754	synaptojanin 2 binding protein (Synj2bp)	1.81	1.68
BG665185	PREDICTED: similar to FLJ44299 protein	2.02	1.58
NM_145789	PREDICTED: similar to isopentenyl-diphosphate delta isomerase 2 (LOC689815)	2.08	1.58
AW524480	AW524480 UI-R-BO0-ahx-h-12-0-UI.s1 UI-R-BO0 Rattus norvegicus cDNA clone UI-R-BO0-ahx-h-12-0-UI 3¢	1.60	1.53
NM_001136470	ubiquitin-fold modifier 1 (Ufm1)	1.83	1.51
	Greater expression for MD		
AF106937	Serine/threonine-protein kinase SIK1 (Salt-inducible protein kinase 1	4.93	1.98
NM_016999	cytochrome P450, family 4, subfamily b, polypeptide 1 (Cyp4b1)	4.86	3.56
NM_139192	stearoyl-Coenzyme A desaturase 1 (Scd1)	2.75	1.51
NM_001115043	similar to Protein C8orf4 (Thyroid cancer protein 1) (TC-1) (LOC684871)	2.56	1.70
NM_138526	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*) (Ccrn4l)	2.55	3.05
NM_134331	Eph receptor A7 (Epha7)	2.42	3.44
NM_030838	solute carrier organic anion transporter family, member 1a5 (Slco1a5)	2.22	2.46
NM_012730	cytochrome P450, family 2, subfamily d, polypeptide 2 (Cyp2d2)	2.01	2.04
BI278569	UI-R-CW0-bxn-c-09-0-UI.s1 UI-R-CW0 *R. norvegicus* cDNA clone UI-R-CW0-bxn-c-09-0-UI 3¢	1.88	1.67
BQ200163	BQ200163 UI-R-DQ1-clu-a-13-0-UI.s1 UI-R-DQ1 *R. norvegicus* cDNA clone UI-R-DQ1-clu-a-13-0-UI 3¢	1.85	1.68
NM_001106615	actin related protein 2/3 complex, subunit 4 (Arpc4)	1.70	2.02
NM_001008321	growth arrest and DNA-damage-inducible, beta (Gadd45b), mRNA [NM_001008321]	1.58	1.88
NM_013071	opioid receptor, mu 1 (Oprm1)	1.34	1.06
NM_053656	purinergic receptor P2X, ligand-gated ion channel, 2 (P2rx2)	1.07	1.17

Genes were selected according to their *P* value (*t*-test; $P < 0.05$) either for the population effect on J or C. Genes were sorted by upregulated in GB woodrats and upregulated in MD woodrats and by fold change.

diet based on the greater level of dispersion of the data for the creosote diet (Fig. 2*B*).

Effect of diet. The greatest number of significant differences in gene expression originated from the effect of diet on a given population, rather between the populations on a given diet. The volcano plots revealed more significant values for a diet effect compared with population differences (Fig. 3, *A* and *B* vs. *C* and *D*, respectively). Great Basin woodrats differentially expressed 7% of the total transcripts on the creosote vs. juniper treatment, while gene expression in Mojave woodrats differed by 12% on creosote vs. juniper treatments (*t*-test, $P < 0.05$, Table 1). When the differences were further dissected within each population, animals on the novel diet treatment expressed a greater proportion of the differentially expressed transcripts (Fisher's exact test, $P < 0.0001$; Table 1). Great Basin woodrats upregulated 38% more genes on a creosote diet relative to their typical diet of juniper, and Mojave woodrats upregulated 43% more genes on juniper compared with their usual diet of creosote. In addition, Mojave population had absolutely more differences in the total number of genes expressed between juniper and creosote compared with Great Basin animals (Fig. 3, *A* and *B*).

Comparison of population responses. Population differences on a given diet were less marked than the diet effect on a given population. These population differences on a given diet were mostly characterized by slight changes in gene expression. This pattern is highlighted by the volcano plots where the scatter plots are more compacted around the origin (Fig. 3, *C* and *D*) than for the diet effect comparisons (Fig. 3, *A* and *B*) where more values were above the criteria for significance (i.e., fold change and *P* value threshold). There was only a 4% difference in number of genes expressed differentially between populations on the creosote diet and a 3% difference between populations on the juniper diet (Table 2). Within a diet treatment, the population for which the diet was novel displayed a significantly greater proportion of upregulated transcripts (Fisher's exact test, $P < 0.0001$; Table 2). That is, the creosote diet induced a higher proportion of upregulated transcripts overall in the Great Basin woodrats, whereas the juniper diet induced a greater proportion of upregulated transcripts in the Mojave woodrats. The asymmetry of volcano plots supported the same conclusion (see Fig. 3, *C* and *D*, respectively).

Some transcripts had similar expression patterns between the two populations, regardless of diet. This intrinsic pattern of gene expression consisted of 22 transcripts for which Great Basin that had greater expression compared with Mojave woodrats and of 14 genes that were upregulated by Mojave woodrats compared with Great Basin, either fed juniper or creosote (Table 3). No GO term was significantly enriched in this small gene list. Note that, compared with Great Basin individuals, Mojave Desert woodrats always upregulated two cytochromes P450 (CYP2D2 and CYP4B1) and a stearoyl-Coenzyme A desaturase 1.

GO terms were screened for overrepresentation in lists of upregulated genes for each population with respect to the novel vs. natural diet. There were 34 GO enriched terms for upregulated genes when Great Basin woodrats were fed creosote compared with juniper (Table 4). When focusing on GO terms at within molecular function (*level 2*), we found enriched terms were heavily represented in the term binding (not shown). Within biological process (*level 2*), enriched terms primarily corresponded to multicellular organismal processes, developmental and metabolic processes (Fig. 4*A*).

Table 4. *GO term enrichment for upregulated transcripts of GB and MD woodrats on a novel diet: 34 enriched GO terms for GB woodrat genes significantly upregulated on the novel diet of C vs. J*

Enriched GO Term	Genes, *n*	% Total	Corrected *P* Value
Binding	**83**	**47.2**	**3.79×10^{-3}**
Extracellular region part	**41**	**23.3**	**3.79×10^{-3}**
Respiratory gaseous exchange	9	5.1	6.36×10^{-3}
Anatomical structure development	11	6.3	1.54×10^{-2}
Nervous system development	10	5.7	1.54×10^{-2}
System development	10	5.7	1.54×10^{-2}
Organic acid metabolic process	4	2.3	1.54×10^{-2}
Cellular amino acid metabolic process	3	1.7	1.54×10^{-2}
Amine metabolic process	3	1.7	1.54×10^{-2}
Extracellular region	**70**	**39.8**	**1.65×10^{-2}**
Ion binding	**58**	**33.0**	**1.65×10^{-2}**
Extracellular space	**41**	**23.3**	**1.65×10^{-2}**
Multicellular organismal development	**25**	**14.2**	**1.65×10^{-2}**
Peptidase inhibitor activity	6	3.4	1.65×10^{-2}
Endopeptidase inhibitor activity	4	2.3	1.65×10^{-2}
Cellular amine metabolic process	3	1.7	1.65×10^{-2}
Oxoacid metabolic process	3	1.7	1.65×10^{-2}
Cellular amino acid and derivative metabolic process	3	1.7	1.65×10^{-2}
Carboxylic acid metabolic process	3	1.7	1.65×10^{-2}
Cellular nitrogen compound catabolic process	1	0.6	1.65×10^{-2}
Cellular amino acid catabolic process	1	0.6	1.65×10^{-2}
Amine catabolic process	1	0.6	1.65×10^{-2}
Cellular ketone metabolic process	3	1.7	1.81×10^{-2}
Developmental process	**28**	**15.9**	**2.77×10^{-2}**
Cation binding	**58**	**33.0**	**3.27×10^{-2}**
Regulation of system process	1	0.6	4.02×10^{-2}
Response to external stimulus	1	0.6	4.37×10^{-2}
Cellular nitrogen compound metabolic process	3	1.7	4.89×10^{-2}
Metal ion binding	**56**	**31.8**	**5.03×10^{-2}**
Cell development	1	0.6	6.29×10^{-2}
Enzyme inhibitor activity	6	3.4	7.07×10^{-2}
Protein K63-linked ubiquitination	2	1.1	7.07×10^{-2}
Regulation of adrenocorticotropin secretion	1	0.6	7.07×10^{-2}
Reproductive structure development	1	0.6	9.37×10^{-2}

Gene Ontology (GO) terms enrichment were screened (GeneSpring GX11 − corrected $P < 0.01$) for the 3 categories within lists of genes that were differentially expressed between diets for a given population (paired *t*-test, $P < 0.05$). Enriched GO terms are ranked by level of significance. The ratio of genes involved in a GO category to the total number of significant genes is displayed in the % Total column. Genes may contribute to several GO terms, i.e., the cumulative % may exceed 100. Terms in which percentages are >10% are highlighted in boldface.

Ninety-seven GO terms were overrepresented in upregulated genes of the Mojave woodrats on the novel diet of juniper vs. creosote. Out of these, 20 terms were represented by at least 10% of genes differentially expressed (note some genes are represented in >1 GO term). These were GO terms that referred mostly to organelle and intracellular components, and binding (Table 5). Enriched terms (*level 2*) mainly corresponded to binding and catalytic activity for the molecular function category (not shown), and to cellular process, and two terms relating to localization for

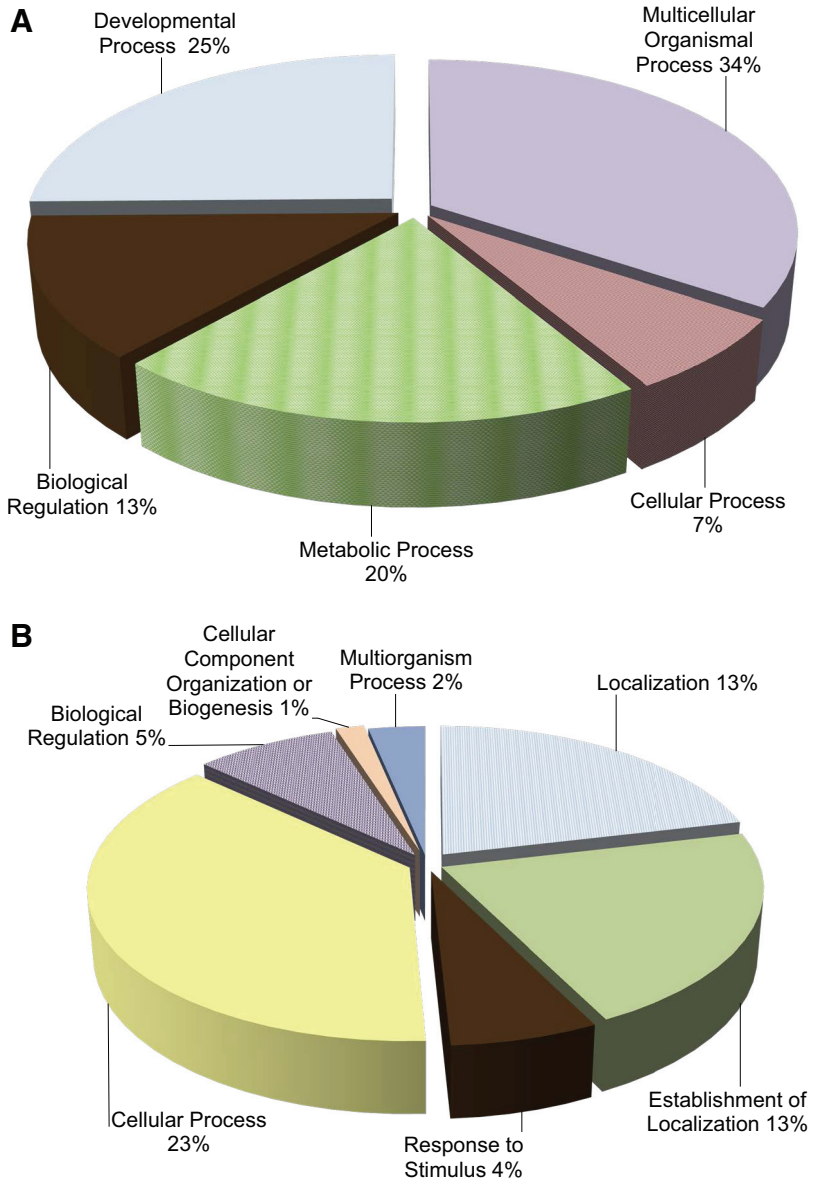

Fig. 4. Pie charts of Gene Ontology (GO) term enrichments for paired comparisons ($P < 0.05$) of changes in gene expression for populations fed a novel diet. Data are presented as level 2 GO categorization for biological process. Classified gene objects are depicted as percentages of the total number of gene objects with GO assignments. *A*: GO analysis of 1,016 transcripts upregulated by GB woodrats when fed the novel C diet compared with the natural J diet. *B*: GO analysis of 1,834 transcripts upregulated by MD woodrats when fed the ancestral J diet of compared with the current C diet.

the biological process category (Fig. 4*B*). Response to stimulus was also notable at 4%.

Diet treatments produced some similar changes in expression between the two populations for subsets of genes. Both populations upregulated the same 282 and 268 genes on creosote and juniper, respectively. These transcripts were sorted according to the gene ontology category. Sixty-three GO terms contained a higher proportion of differentially expressed transcripts than expected just by chance (significant enrichment, corrected $P < 0.01$; Table 6). Categories related to catalytic activity, metabolic process, binding, proteolysis, and oxidoreduction activity (combined across biological process, molecular function, and cellular component).

Contribution of biotransformation processes. Biotransformation genes were present in the upregulated transcripts, but their proportions varied across treatments. Out of the 1,834 upregulated transcripts with known function in Mojave woodrats fed juniper, 20 of these were biotransformation genes. The proportion of biotransformation genes upregulated by Great Basin woodrats on juniper was significantly higher than that found for Mojave woodrats consuming juniper (Fisher's exact test $P > 0.01$; Table 7). In contrast, there was no significant difference between the two populations in the ratio of upregulated biotransformation genes on creosote with respect to the total number of upregulated genes (1.41 and 1.77% for Mojave and Great Basin woodrats, respectively; Table 7).

The proportion of biotransformation genes upregulated by Mojave woodrats was not dependent on diet (Table 8), but diet significantly influenced the proportion of biotransformation genes overexpressed by Great Basin woodrats with proportionally more being upregulated on juniper than creosote (Table 8).

DISCUSSION

Recent advances in molecular approaches facilitate the characterization of candidate biochemical innovations associated

Table 5. *GO term enrichment for upregulated transcripts of GB and MD on a novel diet: 20 GO terms show enrichment for significantly upregulated MD woodrats genes on J vs. C*

Enriched GO Term	Genes, *n*	% Total	Corrected *P* Value
Protein binding	686	66.9	1.32×10^{-17}
Intracellular	672	65.6	1.32×10^{-17}
Intracellular part	612	59.7	3.49×10^{-17}
Binding	801	78.1	2.56×10^{-12}
Purine ribonucleotide binding	169	16.5	4.46×10^{-10}
Ribonucleotide binding	169	16.5	4.46×10^{-10}
Cytoplasm	449	43.8	7.47×10^{-10}
Membrane-bounded organelle	409	39.9	2.32×10^{-9}
Intracellular organelle	407	39.7	2.32×10^{-9}
Purine nucleotide binding	169	16.5	2.32×10^{-9}
Intracellular membrane-bounded organelle	407	39.7	3.03×10^{-9}
Organelle	409	39.9	4.29×10^{-9}
Nucleotide binding	220	21.5	7.92×10^{-9}
Adenyl ribonucleotide binding	169	16.5	1.44×10^{-8}
ATP binding	169	16.5	1.64×10^{-8}
Nucleoside binding	170	16.6	7.58×10^{-8}
Adenyl nucleotide binding	169	16.5	1.01×10^{-7}
Purine nucleoside binding	169	16.5	1.16×10^{-7}
Catalytic activity	169	16.5	1.19×10^{-7}
Nucleus	333	32.5	1.58×10^{-6}
Cytoplasmic part	225	22.0	1.59×10^{-6}
Metabolic process	139	13.6	5.54×10^{-6}
Cytosol	151	14.7	7.62×10^{-5}

GO terms enrichment were screened (GeneSpring GX11 − corrected $P < 0.01$) for the 3 categories within lists of genes that were differentially expressed between diets for a given population (paired *t*-test, $P < 0.05$). Enriched GO terms are ranked by level of significance. The ratio of genes involved in a GO category to the total number of significant genes is displayed in the % Total column. Genes may contribute to several GO terms, i.e., the cumulative % may exceed 100. Categories with percentages >10% are displayed out the overall 97 enriched terms.

with adaptation and diversification in plant-mammal interactions. We took advantage of whole genome microarrays and GO enrichment analyses to explore large-scale patterns of liver gene expression. We found that diet shaped gene expression profiles. Juniper and creosote diets present radically different chemical profiles and influenced gene expression in woodrat livers to a greater extent than population membership. In addition, Great Basin and Mojave woodrats have differential tolerance to creosote and juniper and utilize different detoxification enzymes to biotransform secondary compounds from these plants as a likely result of evolutionary experience (27). Biotransformation processes are energetically costly, but they are less costly than the physiological consequences of toxin ingestion in the absence of detoxification (17). Evolutionary and ecological experience with dietary toxins should select for efficient biotransformation strategies (23), which would lower the physiological and metabolic impact of dietary toxins. This outcome could be manifest in overall gene expression such that relatively fewer genes would be upregulated in animals with evolutionary experience to toxins. In contrast, herbivores lacking evolutionary experience with a particular diet may not have evolved the ability to effectively biotransform PSCs and thus would increase expression of a larger number of genes as they physiologically seek some solution to ingested toxins. The patterns of expression confirmed this hypothesis both from a quantitative and qualitative point of view. Great Basin woodrats, which have no experience with creosote and a lower ability to ingest creosote than Mojave woodrats (27, 29), had relatively greater increase in the number of upregulated genes, many of which were indicative of oxidative stress and cell damage. In contrast, Mojave woodrats, which have evolutionary experience with creosote, upregulated fewer genes, activating cellular processes related to biotransformation strategies.

Effects of reduced food intake. Changes in food intake represent a potential complicating factor in studies of hepatic gene expression. Great Basin woodrats ate less on the creosote diet compared with their counterparts on juniper and also compared with Mojave animals on creosote. This reduced food intake resulted in a 5.9% ± 1.3 reduction in body mass of these animals (27). Previous studies have shown that caloric restriction induces GO enrichment of genes involved in intermediary and energy metabolism (33). Indeed, Great Basin woodrats exhibited enrichment for genes related to energy metabolism on creosote vs. juniper (i.e., carboxylic acid, organic acid, amine, amino acid metabolism, Table 4).

In other studies, dramatic decreases in average daily food intake (55% reduction) coupled with significant changes in body mass obfuscate direct effects of toxins from the secondary effects on the liver associated with decreased food intake (35). In toxicological studies there is a need to distinguish true compound-derived impacts from those resulting from changes in body weight (33). We controlled for large decreases in food intake in our study by removing individuals that exceeded a 10% body mass loss over the trial period ($n = 1$). In addition, the short duration of the dietary treatment (5 days) circumvented secondary inflammatory response or fibrosis commonly found in longer-term trials (40). Thus, the majority of the trends observed in this study are likely due to a direct diet change effect.

Dietary toxins shape liver physiological response. Dietary PSCs had profound effects on overall gene expression in this study, more so than population membership. Juniper and creosote produce disparate plant secondary compounds. Creosote produces >300 natural products, many of which are polyphenolics with the main component of resin being NDGA, a lignan catechol (26). In contrast, terpenes are the primary class of secondary compounds in juniper (1, 34). The primary difference between the juniper and creosote diets is related to types of PSCs. This difference in class of PSCs induced large-scale changes in the physiological response of the liver.

This result is consistent with other work highlighting distinct gene expression patterns when lab rats are exposed to various classes of compounds. Toxicants can even be classified by their gene expression profiles derived from the primary target tissue (blood and/or liver; 4, 12, 24). Juniper and creosote are processed by distinct biotransformation enzymes. Woodrats from Mojave and Great Basin rely heavily on functionalization enzymes in the biotransformation of juniper (i.e., an overexpression of 13 CYP P450 isozymes; Ref. 27). In contrast, for both populations on creosote, multiple enzymes in the glutathione conjugation pathway ("GSTs") were upregulated compared with a juniper diet.

Native vs. novel diets. Overall gene expression of both populations was more similar when woodrats were eating juniper compared with creosote: 49% of upregulated transcripts were shared for biotransformation genes only and 11% of all genes between Mojave and Great Basin woodrats consuming juniper, whereas only 21% for biotransformation and

Table 6. *GO term enrichment for shared population responses to diet change from C to J*

GO Term	Count in Selection	% Count in Selection	Corrected *P* Value
Proteasome complex	17	14.66	7.99×10^{-11}
Negative regulation of ubiquitin-protein ligase activity	15	12.93	1.84×10^{-8}
Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	15	12.93	1.84×10^{-8}
Negative regulation of ligase activity	15	12.93	1.84×10^{-8}
Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	15	12.93	1.84×10^{-8}
Positive regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	15	12.93	2.02×10^{-8}
Regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	15	12.93	2.97×10^{-8}
Positive regulation of ubiquitin-protein ligase activity	15	12.93	3.37×10^{-8}
Positive regulation of ligase activity	15	12.93	4.97×10^{-8}
Negative regulation of protein ubiquitination	15	12.93	7.27×10^{-8}
Proteasomal ubiquitin-dependent protein catabolic process	15	12.93	8.63×10^{-8}
Proteasomal protein catabolic process	15	12.93	8.63×10^{-8}
Regulation of ubiquitin-protein ligase activity	15	12.93	1.12×10^{-7}
Regulation of ligase activity	15	12.93	1.62×10^{-7}
Positive regulation of protein ubiquitination	15	12.93	7.71×10^{-7}
Ubiquitin-dependent protein catabolic process	20	17.24	8.60×10^{-7}
Modification-dependent protein catabolic process	20	17.24	1.29×10^{-6}
Modification-dependent macromolecule catabolic process	20	17.24	1.29×10^{-6}
Negative regulation of cellular protein metabolic process	15	12.93	2.57×10^{-6}
Proteolysis involved in cellular protein catabolic process	21	18.10	2.93×10^{-6}
Negative regulation of catalytic activity	16	13.79	2.93×10^{-6}
Negative regulation of protein modification process	15	12.93	2.93×10^{-6}
Cellular protein catabolic process	21	18.10	3.20×10^{-6}
Negative regulation of protein metabolic process	15	12.93	6.69×10^{-6}
Regulation of protein ubiquitination	15	12.93	1.45×10^{-5}
Protein catabolic process	21	18.10	1.94×10^{-5}
Negative regulation of molecular function	16	13.79	2.26×10^{-5}
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	10	8.62	2.53×10^{-5}
Metabolic process	85	73.28	4.96×10^{-5}
Macromolecule catabolic process	21	18.10	4.96×10^{-5}
Vesicular fraction	24	20.69	6.75×10^{-5}
Cellular macromolecule catabolic process	21	18.10	1.06×10^{-4}
Microsome	23	19.83	1.53×10^{-4}
Catalytic activity	60	51.72	1.90×10^{-4}
Negative regulation of metabolic process	16	13.79	5.16×10^{-4}
Aromatase activity	8	6.90	5.16×10^{-4}
Cell cycle process	15	12.93	5.17×10^{-4}
Catabolic process	21	18.10	5.20×10^{-4}
Cell cycle	22	18.97	6.15×10^{-4}
Cellular catabolic process	21	18.10	6.65×10^{-4}
Positive regulation of protein modification process	15	12.93	6.74×10^{-4}
Negative regulation of macromolecule metabolic process	15	12.93	7.00×10^{-4}
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	10	8.62	8.04×10^{-4}
Negative regulation of cellular metabolic process	15	12.93	1.05×10^{-3}
Oxidation reduction	29	25.00	1.80×10^{-3}
Mitotic cell cycle	15	12.93	1.80×10^{-3}
Nuclear membrane-endoplasmic reticulum network	22	18.97	2.12×10^{-3}
Organic acid metabolic process	1	0.86	2.12×10^{-3}
Proteolysis	32	27.59	2.21×10^{-3}
Proteasome core complex	6	5.17	2.21×10^{-3}
Binding	40	34.48	2.24×10^{-3}
Oxidoreductase activity	30	25.86	2.31×10^{-3}
Threonine-type peptidase activity	6	5.17	2.87×10^{-3}
Threonine-type endopeptidase activity	6	5.17	2.87×10^{-3}
Cell fraction	26	22.41	3.22×10^{-3}
Monooxygenase activity	11	9.48	3.30×10^{-3}
Endoplasmic reticulum membrane	22	18.97	3.35×10^{-3}
Gamete generation	4	3.45	4.32×10^{-3}
Proteasome core complex, alpha-subunit complex	4	3.45	5.18×10^{-3}
Testosterone 6-beta-hydroxylase activity	3	2.59	6.30×10^{-3}
Peptide disulfide oxidoreductase activity	1	0.86	6.30×10^{-3}
Heme binding	13	11.21	7.52×10^{-3}
Endoplasmic reticulum part	22	18.97	8.12×10^{-3}

GO terms enrichment were screened (GeneSpring GX11 − corrected $P < 0.01$) for the 3 categories within lists of genes that were differentially expressed between diets for both populations (paired *t*-test, $P < 0.05$). Enriched GO terms are ranked by level of significance. The ratio of genes involved in a GO category to the total number of significant genes is displayed in the % Total column. Genes may contribute to several GO terms.

Table 7. *Genes differentially expressed on J vs. C diets*

Expression Pattern	Transcript Category	MD	GB	Difference in Proportion
Upregulated on J	known function	1,834	735	***P* = 0.0042**
	biotransformation	20	20	
	ratio (%)	1.09	2.72	
Upregulated on C	known function	1,275	1,016	$P = 0.5036$
	biotransformation	18	18	
	ratio (%)	1.41	1.77	

Pairwise comparisons in gene expression were established for biotransformation and known gene lists Numbers displayed correspond to significant differences in expression of unique genes (raw P value < 0.05; fold change > 1.5). The 4 types of pairwise t-tests aimed at characterizing the impact of population membership either on gene expression patterns on C or J. Significant differences in proportions of biotransformation genes are highlighted in boldface (Fisher's exact test, $P > 0.01$).

14% of all genes were shared between the two populations when they consumed creosote. This higher level of similarity on juniper may be the result of shared evolutionary experience of both Mojave and Great Basin woodrats with *J. osteosperma*, whereas the Great Basin animals are naïve to creosote. Mojave woodrats, although they lack current ecological exposure to juniper, have historical experience this dietary component, and they appear to have retained some of the physiological responses to juniper.

Diet treatments induced a more substantial difference in the pattern of gene expression for Mojave woodrats than for Great Basin animals. Mojave woodrats differentially regulated absolutely more transcripts across the two diet treatments overall (3,109 vs. 1,751 for Great Basin). This may reflect the fact that Mojave individuals had prior experience with both diets and thus developed a distinct strategy for each type of PSC. The Mojave population appears to have evolved a different biotransformation response to its current diet of creosote compared with the Great Basin population for which creosote is novel. For example, with respect to biotransformation genes only, the Mojave population fed creosote had elevated expression of an additional seven probes for enzymes related to the glutathione pathway (27). This suggests a unique biotransformation strategy appeared over the course of Mojave woodrat evolution in response to invading creosote to produce a different set of biotransformation enzymes for metabolizing creosote. The large differences in gene expression between these populations warrant further investigation into the possible causes.

The ratio of upregulated biotransformation genes to the total number of genes appears to be related to previous experience with the diet. In those cases where the population had previous ecological or evolutionary experience with the diet, biotransformation genes represented a greater proportion of upregulated genes overall. This ratio reflects the biotransformation investment compared with the overall physiological consequence of the diet. This pattern of relatively more biotransformation genes on both diets for Mojave woodrats could result from this population's evolutionary experience with both sets of toxins, i.e., creosote presently and juniper historically. In contrast, Great Basin woodrats had no previous experience with creosote and had fewer upregulated biotransformation genes relative to all genes on creosote compared with juniper. Although there was no difference in the proportion of upregulated biotransformation genes in Mojave woodrats on the two diets, Great Basin woodrats expressed a greater proportion of detoxification genes on juniper compared with Mojave woodrats on juniper. Thus, although the Mojave population appears to retain the ability to ingest similar quantities of juniper compared with the Great Basin population, notable differences existed with respect to expression of biotransformation genes between the two populations when consuming juniper.

Biotransformation ability and metabolic benefit. Gene modulation of cellular activities corresponds to a wide variety of functions. Unfortunately, for the large majority of these loci, we know very little of their biology, yet general patterns of expression can be interpreted thanks to GO class enrichment: the integrated profile of this subset of transcripts discriminates between the different treatments more robustly than any one candidate gene alone (39).

A common trend was observed for the two populations when the ratios of upregulated gene were compared between creosote and juniper diets. Both ecotypes upregulate relatively more genes on the more novel diet than on the currently ingested diet, but fewer of these upregulated genes are biotransformation genes. One interpretation of this pattern is that the animals may not be able to marshal the appropriate detoxification genes toward the novel toxins, hence more genes with general functions were upregulated to either prevent or respond to the physiological consequences resulting from the ingestion of novel PSCs. We recognize that validation of this phenomenon will require further study. However, evidence from the literature is consistent with this interpretation. Energy metabolism has been documented to increase in sheep when toxins are administered in a way that bypasses the liver (18). A change in energy metabolism would likely result in concomitant changes in liver enzyme transcripts. Furthermore, Grbic et al. (10) found that more genes were differentially expressed for spider mites confronted with hosts to which it is not adapted, relative to their preferred host. Genes in the detoxification and peptidase families exhibited the most profound changes in expression.

PSC metabolism results in the production of organic acids that can challenge acid-base homeostasis. The elimination of hydrogen ions (H^+) from organic acids occurs through various ways including their reaction with bicarbonate in the extracellular fluid to form carbon dioxide that is then exhaled (8). Out

Table 8. *Genes differentially expressed for MD vs. GB woodrat populations*

Expression Pattern	Transcript Category	C	J	Difference in Proportion
Upregulated by MD	known function	397	481	$P = 0.1296$
	biotransformation	10	6	
	ratio (%)	2.52	1.25	
Upregulated by GB	known function	702	268	***P* = 0.0073**
	biotransformation	1	5	
	ratio (%)	0.14	1.87	

Pairwise comparisons in gene expression were established for biotransformation and known gene lists Numbers displayed correspond to significant differences in expression of unique genes (raw P value < 0.05; fold change > 1.5). The 4 types of pairwise t-tests aimed at characterizing the effect of diet treatment on gene expression in MD and GB woodrats populations. Significant differences in proportions of biotransformation genes are highlighted in boldface (Fisher's exact text, $P > 0.01$).

of the 34 GO terms showing enrichment for Great Basin-upregulated transcripts on creosote vs. juniper, more than one-third are consistent with this observation: seven terms relate to amine and nitrogen metabolism, three are extracellular region processes, and an additional four deal with organic acid, oxoacid, cation metabolic processes, or respiratory gaseous exchange. We hypothesize that these groups of upregulated genes are in response to the physiological consequences of creosote PSCs on animals that have no evolutionary experience with these toxins. In the same way, oxidative stress possibly related to the lack of an appropriate biotransformation mechanism may explain the enrichment in seven terms related to cell metabolism and development in Great Basin woodrats on creosote (Table 4). Indeed, Dragin et al. (6) showed that oxidative stress triggers the activation of regeneration-related events in the liver.

In contrast, we do not find enrichment in such terms among the 97 that were overrepresented by Mojave woodrats on the unusual diet of juniper vs. creosote (Table 5). Thirty terms can directly be attributed to intracellular and organelle activities, whereas approximately the same quantity refer to protein or macromolecules, including ubiquitin, which plays a role in protein tagging for further degradation. While Mojave woodrats do not have ecological experience with juniper, it was their ancestral diet 17,000 yr ago. Thus, these animals seem to retain the ability to upregulate genes appropriate for the metabolism of these compounds.

Conclusion

The application of transcriptomic approaches permits investigation into the strategies that may arise through herbivore-plant coevolution. The study presented herein found an interesting pattern of gene expression to be tested in future more reductionist experiments. Herbivores with no previous experience with a diet appear to respond to a novel diet through the upregulation of relatively more transcripts either to reduce the damage of the novel compound or in response to damage caused by a novel compound. Furthermore, animals with evolutionary but not ecological experience appeared to retain the ability to metabolize an ancient diet.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.M. and J.M. performed experiments; E.M. analyzed data; E.M. and M.D.D. interpreted results of experiments; E.M. prepared figures; E.M., J.M., and M.D.D. drafted manuscript; E.M., J.M., and M.D.D. edited and revised manuscript; E.M. and M.D.D. approved final version of manuscript; M.D.D. conception and design of research.

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