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Expression of biotransformation genes in woodrat (*Neotoma*) herbivores on novel and ancestral diets: identification of candidate genes responsible for dietary shifts

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Abstract

The ability of herbivores to switch diets is thought to be governed by biotransformation enzymes. To identify potential biotransformation enzymes, we conducted a large-scale study on the expression of biotransformation enzymes in herbivorous woodrats (*Neotoma lepida*). We compared gene expression in a woodrat population from the Great Basin that feeds on the ancestral diet of juniper to one from the Mojave Desert that putatively switched from feeding on juniper to feeding on creosote. Juniper and creosote have notable differences in secondary chemistry, and thus, should require different biotransformation enzymes for detoxification. Individuals from each population were fed juniper and creosote diets separately. After the feeding trials, hepatic mRNA was extracted and hybridized to laboratory rat microarrays. Hybridization of woodrat samples to biotransformation probes on the array was 87%, resulting in a total of 224 biotransformation genes that met quality control standards. Overall, we found large differences in expression of biotransformation genes when woodrats were fed juniper vs. creosote. Mojave woodrats had greater expression of 10× as many biotransformation genes as did Great Basin woodrats on a creosote diet. We identified 24 candidate genes that may be critical in the biotransformation of creosote toxins. Superoxide dismutase, a free radical scavenger, was also expressed to a greater extent by the Mojave woodrats and may be important in controlling oxidative damage during biotransformation. The results are consistent with the hypothesis that biotransformation enzymes limit diet switching and that woodrats in the Mojave have evolved a unique strategy for the biotransformation of creosote toxins.

Keywords: biotransformation, creosote, detoxification, juniper, woodrats

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Introduction

For herbivorous mammals, food ingestion can have deleterious consequences. At every meal, herbivores confront defensive compounds in plants that are potentially toxic. Some herbivores such as Stephen's woodrat (*Neotoma stephensi*) have evolved the ability to specialize on plant species with high concentrations of plant secondary compounds (PSC), whereas other herbivores consume many species of plants with lower concentrations of different PSCs, presumably to keep toxin concentrations at levels with minimal physiological impacts (Freeland & Janzen 1974; Vaughn 1982; Marsh *et al*. 2006). The diet breadth of herbivores as well as the ability to adapt to new dietary components is thought to be governed by biotransformation ('detoxification') enzymes in the liver (Klaassen 2001). Hundreds of biotransformation enzymes critical for drug metabolism have been documented for laboratory rats (Klaassen 2001; Martignoni *et al*. 2006). However, very little is known about the specific biotransformation enzymes employed by mammalian herbivores, especially

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in comparison to their insect counterparts (Dearing *et al*. 2005; Li *et al*. 2007).

The desert woodrat, *Neotoma lepida*, is an exemplary system to examine hypotheses related to the biotransformation of PSCs and diet shifting. At the end of the Pleistocene (18 700–10 000 years ago), woodrats occupying the southwestern USA and northern Mexico underwent a major dietary shift and began feeding on a natural invader, creosote, *Larrea tridentata* (Van Devender 1977; Van Devender & Spaulding 1979; Hunter *et al*. 2001). Data from fossilized woodrat middens in southwestern California, Baja California, Mexico and southwestern Arizona indicate that woodrats collected and presumably fed on creosote in the Late Pleistocene (Van Devender 1990). Currently, creosote can constitute up to 75% of the diet of woodrats in the Mojave Desert (Karasov 1989). Prior to the invasion of creosote and later establishment of the Mojave desert in the Holocene, juniper woodlands (*Juniperus* spp.) were widespread in the southwest (Van Devender 1977; Van Devender & Spaulding 1979). Juniper was present in woodrat middens and began to disappear from middens at lower altitudes in this region during its extirpation in the Holocene (Van Devender 1990; Van Devender & Spaulding 1979). Thus, it is plausible that the woodrat populations in the Mojave Desert that currently feed on creosote had an ancestral diet containing juniper. Closely related, extant populations of *N. lepida*, outside of the Mojave, continue to feed on juniper. This putative switch from a diet of juniper to that of *L. tridentata* represents a marked change in the types of dietary toxins ingested. Creosote and juniper are radically different with respect to profiles of PSCs. Juniper contains numerous terpenes (> 35 monoterpenes) that can constitute up to 5% of the dry weight as well as less abundant tannins (Schwartz *et al*. 1980; Adams *et al*. 1981; Nunez-Hernandez *et al*. 1989). Terpenes depress the central nervous system. In contrast, creosote leaves are coated with a complex resin comprised of numerous polyphenolic compounds (Mabry *et al*. 1977). Resin content of the leaves can vary from 10 to 25% (dw). The primary component of resin is nordihydroguaiaretic acid (NDGA), which has detrimental effects when fed to laboratory rats (Grice *et al*. 1968; Goodman *et al*. 1970).

The creosote bush invasion did not extend through the entire range of the desert woodrat. In the Great Basin desert, populations of desert woodrats still feed on juniper (*Juniperus osteosperma*). These woodrats have no evolutionary or ecological experience with creosote. The populations of woodrats in the Great Basin and Mojave are closely related and are thought to have diverged only within the past 60 000 years (Patton *et al*. 2008). These closely related populations of woodrats that feed on juniper vs. those that consume a novel diet of creosote permit investigations into the evolutionary traits that facilitate creosote feeding.

Populations of desert woodrats that currently live in the Mojave (herein 'Mojave woodrats') have adjusted to a diet of creosote as evidenced by their ability to ingest greater quantities of creosote resin (25% more) compared to Great Basin desert populations naïve to creosote (Mangione *et al*. 2000, 2001). This difference in tolerance between Mojave and Great Basin woodrats appears to be caused by differential abilities to biotransform creosote toxins. Previous studies on the hepatic biotransformation pathways used by Mojave and Great Basin woodrats are consistent with differential biotransformation (Mangione *et al*. 2001; Haley *et al*. 2008). However, a limitation of these studies is that they examined only a handful of prospective pathways (8 total), whereas mammals possess hundreds of enzymes that participate in the biotransformation of xenobiotics (Klaassen 2001; Martignoni *et al*. 2006).

To more thoroughly examine the biotransformation differences between Mojave and Great Basin woodrats, we took advantage of recent technological advances that permit the simultaneous investigation of hundreds of biotransformation enzymes. We used microarrays designed for laboratory rats to investigate the comparative expression of hepatic biotransformation enzymes in woodrats on different diets. Woodrats and laboratory rats, being murid rodents, are sufficiently related to permit the use of laboratory rat microarrays on woodrat samples (Skopec *et al*. 2007). Using microarrays, we tested the hypothesis that herbivores employ distinct biotransformation pathways to metabolize plants with different secondary compound profiles. We also explored the hypothesis that Mojave woodrats consuming creosote utilize hepatic biotransformation enzymes that differ from closely related woodrat populations naïve to creosote that still feed on juniper, the putative ancestral diet of Mojave woodrats. Lastly, we examined whether Mojave woodrats use the same pathways to metabolize their putative ancestral diet of juniper as those used by the Great Basin woodrats that currently feed on juniper.

Materials and methods

Woodrat collection

Woodrats (*Neotoma lepida*) from the Mojave Desert were trapped near Beaver Dam (Lytle Ranch), in Washington County, Utah (37°07′N, 114°00′W) on 6–8 December 2006. We used Sherman live traps baited with peanut butter and oats; cotton batting was provided for nesting material. The vegetation of the Beaver Dam site primarily consisted of creosote bush (*Larrea tridentata*), black bush (*Coleogyne ramosissima*), Joshua tree (*Yucca breviflora*), desert almond (*Prunus fasciculata*) and cholla (*Cylindropuntia* spp.). We collected creosote foliage for use in the feeding trials and stored it at –20° until use.

Woodrats from the Great Basin were trapped on 7–8 February 2007 near White Rocks, Tooele County, Utah (40°19′N, 112°54′W). At the White Rocks site, juniper (*Juniperus*

osteosperma) was the dominant tree and big sagebrush (*Artemisia tridentata*) was the dominant shrub. Creosote bush is not present in this habitat. Juniper foliage was collected in Little Cottonwood Canyon, Salt Lake County. Foliage was kept on dry ice after collection until transport to a –20 °C freezer where it was stored in closed plastic bags until use.

We confirmed that the two populations were from the same subclade (2 A) as defined by Patton *et al*. (2008) by sequencing an 800 + bp portion of the mitochondrial DNA cytochrome *b* gene for three individuals per location. Sequences were corrected by eye, and aligned in Sequencher (GeneCodes) and compared to sequences available in GenBank for *N. lepida* (Accession nos DQ179830–DQ179838; DQ781146–DQ781166; DQ781250–DQ781253; and DQ781296–DQ781305) with Molecular Evolutionary Genetics Analysis (MEGA), version 3.1 (Kumar *et al*. 2004). *Neotoma stephensi* was used as an outgroup (GenBank Accession no. DQ781305). The aligned sequences were analysed by distance (neighbour joining: Saitou & Nei 1987), using the Kimura 2-parameter distance estimator). The robustness of inferences was assessed by bootstrap resampling (500 random repetitions (Felsenstein 1985)). Five haplotypes were identified among our 6 *N. lepida* cytochrome b sequences, but the differentiation was low: the complete data matrix comprised 722 base pairs, of which only 19 sites (2.6%) were variable. The phylogenetic reconstruction by distance using GenBank sequences in addition to our matrix provided a phylogenetic tree with the same topology as the one proposed by (Patton *et al*. 2008). The results confirmed that both the Great Basin and Mojave populations were from clade 2 A (bootstrap support: 92%).

Animal housing and diet preparation

All the woodrats were transported to the University of Utah, Department of Biology's Animal Facility. Woodrats were housed in individual cages (48 × 27 × 20 cm) and kept in quarantine until being screened for hantavirus (Dearing *et al*. 1998). No animals tested positive for hantavirus. Woodrats were acclimated to captivity (12:12 light:dark cycle, 22–28 °C, 15% humidity) for 3 months before the experiment. Prior to feeding trials, woodrats were fed standard rabbit chow (Harlan Teklad formula 2031). Water was provided *ad libitum*. All experimental procedures were approved by the University of Utah's Institutional Animal Care and Use Committee (protocol no. 07-02015).

Creosote resin was extracted by soaking leaf tissue in acetone for 45 min (1:6 wet weight:acetone volume). The extract was filtered (Whatman no. 4 paper) and evaporated under low pressure until the resin was highly viscous. Remaining solvent was removed by drying the extract to constant mass under high vacuum (10^{-3} Torr) for ~48 h. The extraction procedure yielded 18.9% powdered creosote resin by dry weight of creosote leaves. The resin was stored at –20 °C for less than three months before use.

The creosote diet treatments consisted of 1% and 2% resin. We selected these concentrations based on a pretrial to determine the maximum concentration of creosote that could be tolerated by Great Basin woodrats without significant mass loss (i.e. < 10% per night). Treatments were prepared by dissolving the desired amount of powdered resin into acetone, and applying the acetone-resin mixture to ground rabbit chow (Harland Teklad formula 2031) using a volume equal to 25% of the dry weight of chow. Acetone was evaporated from diet treatments in a fume hood. The diet was then placed under high vacuum (10^{-3} Torr) for 2 h to remove any remaining solvent. Complete evaporation was confirmed gravimetrically and dry diet treatments were stored at –20 °C.

The juniper diet treatments consisted of 10%, 20%, 30% and 50% juniper foliage by dry weight added to ground rabbit chow. These concentrations were determined in previous feeding trials with 50% juniper representing the maximum tolerable concentration of juniper foliage for both populations (M. Dearing, unpublished). Foliage was crushed on dry ice to produce plant fragments that were < 0.1 mm. Ground juniper was stored in plastic bags at –20 °C until the day of use. Juniper treatments were prepared daily from the frozen foliage. For consistency with the creosote treatments, the rabbit chow to which the juniper was added was pretreated with acetone (25% by dry weight); acetone was evaporated using the same process as described for the creosote diet.

Feeding trials

The creosote and juniper treatments were fed to eight woodrats from the Mojave (six males, two females) and eight woodrats from Great Basin (four males, four females). After a 4-day pretrial to determine food intake on a nontoxic diet of rabbit chow, 4 woodrats from each population were exposed to either increasing levels of creosote resin or increasing levels of juniper in the diet over a 5-day trial. There was no difference in body mass between the four experimental groups (137.4 ± 5.6 g; $F_{3,12} = 0.3$, $P = 0.8$).

All feeding trials began with acclimation periods where animals were first exposed to lower levels of toxins to permit induction of biotransformation enzymes prior to being given the maximum tolerable dose. The juniper feeding trial consisted of a 2-day period on 10% juniper, followed by a day on 20%, a day at 30%, and a day at 50% juniper. The creosote trial consisted of a 2-day period on 1% resin, followed by 3 days on 2% resin. These levels of resin in the treatment simulated a diet of 19% and 38% creosote. Diet treatments and water were provided *ad libitum*. Diets were presented daily 1 h before dark each day for a 24-h period.

All leftovers were collected and dried to determine daily food intake. Body mass was measured each day.

At the end of the feeding trials, woodrats were euthanized via CO_2 asphyxiation. Euthanization occurred about 5 h into light phase, thus at least 5 h had passed since the final meal. Livers were removed for the microarray analyses weighed and cut into subsamples (~20 mg) and incubated overnight at 4 °C in RNA Later (Ambion, Inc.). The liver samples were removed from the solution and frozen at –80 °C.

To determine whether woodrat populations (Great Basin vs. Mojave) within a dietary treatment differed in food intake, toxin intake, or change in body mass, we used one-way analyses of variance (ANOVA). Within a diet treatment, population was the independent variable and either food intake, toxin intake (Day 5 or Cumulative), liver mass, or change in body mass was the dependent variable in each ANOVA (SYSTAT version 10). Because only two populations were compared within each ANOVA, e.g. food intake of Great Basin vs. Mojave on a creosote diet, *post hoc* analyses were not applicable. All data were checked for normality prior to the analyses. The distributions of these variables did not deviate from a normal distribution; hence, no transformations were applied.

RNA preparations

Total RNA from the 20-mg liver sections was extracted using Tri Reagent (Sigma) per the manufacturer's protocol. The samples were purified with a DNase treatment from RNaqueous-4PCR (Ambion, Inc.). The quality of the RNA was assessed using an RNA Monochip Bioanalyzer system (Agilent Technologies) and quantity was determined using a Nanodrop ND-1000 spectrophotometer. Total RNA (500 ng/sample) was labelled 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 using the Agilent Gene Expression Hybridization Kit following the manufacturer's instructions at the Huntsman Cancer Institute Microarray Core Facility at the University of Utah.

Microarray experiments

The fluorescently labelled amplified RNA samples were hybridized to Agilent Technologies 60mer oligonucleotide rat 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 (1 per woodrat within a diet treatment and a population) were used with a reference design. A common reference of total RNA pooled from an equal amount of the 16 samples was used. Following hybridization, the gene 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.

Quantitative PCR verification

Quantitative real-time polymerase chain reaction (qPCR) was conducted on superoxide dismutase (SOD), cytochrome P450 2A3A (CYP2A3A) and P450 (cytochrome) oxidoreductase (POR) to verify the microarray results. RNA from the above microarray experiment was treated with DNaseI (Fermentas), and cDNA was generated with an Enhanced Avian HS RT-PCR Kit (Sigma), following the manufacturer's protocol. Quantitative PCR was performed using a Roche Lightcycler 2.1. Gene-specific primers were designed with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were as follows: SOD forward 5′-CACTTCGAGCAGAAGGCAAG-3′ and reverse 5′-CACCTTTGCCCAAGTCATCT-3′; CYP2A3A forward 5′-GACCGAATGAAGATGCCCTA-3′ and reverse 5′-GGAAGTGCTTTGGGTTGAAG-3′; and POR forward 5′-GAYGACGGGAACTTGGAAGA-3′ and reverse 5′-ACCTTGGCTRYGTCCATGTC-3′. Primers were also designed for β-actin, the housekeeping gene used to normalize target gene expression (forward 5′GTCCCTGTATGCCTCTGGTC3′ and reverse 5′GCTGTGGTGGTGAAGCTGTA3′). β-actin was chosen as a normalizer gene because it has been shown to be consistent in expression in previous studies of liver enzyme expression in rodents (see Bartosiewicza *et al*. 2000). In addition, we tested β-actin for its fitness as a normalizer gene in this study by comparing β-actin copy number across treatments using subsamples diluted to equal concentrations of cDNA (ng/μL). Each qPCR was 10 μL:1 μL of mixed forward and reverse DNA oligo primers (5 μM each), 2 μL 5× Master Mix (LightCycler FastStart DNA MasterPLUS SYBR Green I, Roche), 4.5 μL PCR-grade water and 2.5 μL of cDNA (50–100 ng/μL). Cycling conditions were: pre-incubation 95 °C, 10 min followed by 35 amplification cycles (95 °C, 10 s; 61 °C, 5 s; 72 °C, 15 s) and a 1-min extension at 72 °C. All reactions were performed in duplicate with samples from all four replicates for each diet and population treatment. The formation of a single PCR product was confirmed using the melting curves and visualization of product on 1% agarose gel. The expression ratios of SOD, CYP2A3A and POR to the housekeeping gene were calculated and compared to the microarray results.

Table 1 Means ± 1 SE for variables measured in the feeding trials of Mojave and Great Basin woodrat populations (*Neotoma lepida*) fed treatments containing either creosote resin or juniper foliage. Body mass represents the mass at the start of the trials. Intakes (Day 5 and Cumulative) are calculated for either the amount of creosote resin or amount of juniper ingested for a particular trial. Within a diet treatment, different letters indicate significant differences between populations (ANOVA)

Diet	Population	Body mass (g)	% change in body mass	Day 5: intake (g)	Cumulative intake (g)	Liver mass (g)
Creosote	Great Basin	139.0 ± 13.5 a	–5.9 ± 1.3 a	0.16 ± 0.02 a	0.71 ± 0.07 a	3.88 ± 0.64 a
	Mojave	145.8 ± 11.3 a	2.9 ± 0.5 b	0.23 ± 0.02 b	0.93 ± 0.05 b	4.73 ± 0.73 a
Juniper	Great Basin	135.8 ± 8.8 a	–4.8 ± 0.7 a	3.1 ± 0.3 a	12.1 ± 1.0 a	5.03 ± 0.51 a
	Mojave	128.3 ± 13.7 a	–3.4 ± 1.0 a	2.6 ± 0.2 a	10.1 ± 0.7 a	4.13 ± 0.52 a

Data analysis

We limited our analyses in this study to the probes on the array related to the metabolism of xenobiotics. Of the 32 818 probes on the array for unique genes with known function, we identified 259 unique genes represented by 295 unique probes that corresponded to biotransformation enzymes. Approximately 65% of the probes represented functionalization enzymes, whereas the remaining 35% were conjugation enzymes.

Statistics: to determine the overall variation in gene expression between treatments, we conducted a principal component analysis (PCA; SYSTAT version 10). Only ratios of genes with signal intensities > 1 for all individuals were included in the PCA.

We probed the differences in expression of biotransformation genes between population and diet treatments with three sets of *T* tests. First, to determine whether the suites of biotransformation enzymes differed between juniper and creosote, we compared expression between diets for each population independently with *T* tests. Second, we tested the hypothesis that Mojave woodrats have evolved a novel process for the biotransformation of creosote by comparing expression of biotransformation genes of both populations on the creosote diet using *T* tests. Third, to determine whether Mojave woodrats retain similar usage of biotransformation enzymes on their putative ancestral diet, we compared gene expression of both populations on juniper. Individual transcripts were included when at least three individuals per population had a signal intensity > 1. Intensity data for each gene were $\log_2$-transformed before statistical analysis. To identify lists of candidate biotransformation genes from these sets of *T* tests, we rank ordered genes from highest to lowest based on fold-change in expression for genes that were significant at $P < 0.05$ (Guo *et al*. 2006; Kuo *et al*. 2006). We acknowledge that this is a more liberal statistical approach than some of the others that adjust for multiple comparisons (e.g. Storey's Q, $P < 0.001$ (Storey & Tibshirani 2003)). However, we feel that this approach was the most appropriate because the fold-change in expression is more consistent across laboratories and platforms (Guo *et al*. 2006). 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 such as this one.

Results

Creosote feeding trial

The Great Basin and Mojave woodrats differed in performance on the creosote resin trial. The Great Basin woodrats were in negative mass balance after the feeding trial, whereas the Mojave woodrats were in positive mass balance ($F_{1,6} = 22.7$, $P = 0.002$, Table 1). The cumulative amount of resin ingested during trial differed between the two populations ($F_{1,6} = 6.6$, $P = 0.043$). Mojave woodrats ingested ~30% more resin over the 5-day period. However, the difference in intake on the last day of the feeding trial was only marginally significant ($F_{1,6} = 5.3$, $P = 0.06$). There was no difference in liver mass between the two populations at the end of the feeding trial ($F_{1,6} = 0.8$, $P = 0.42$; Table 1).

Juniper feeding trial

There was no difference in performance of the Great Basin vs. the Mojave woodrats on the juniper diet treatment. Both populations were in similar negative mass balance by the end of the trial ($F_{1,6} = 1.4$, $P = 0.28$, Table 1). Woodrats did not differ in the cumulative consumption of juniper across the trial ($F_{1,6} = 1.6$, $P = 0.25$); nor did they differ in juniper consumption on the last day of the trial ($F_{1,6} = 2.8$, $P = 0.15$; Table 1). There was no difference in liver mass between the two populations ($F_{1,6} = 1.1$, $P = 0.27$, Table 1).

Quality of interspecific hybridization and overall pattern

Of the 32 818 unique genes with known function on the array, 72% had an average quality control index of 0.75, i.e. three of four individuals had quality control index of 1. Of the 259 unique biotransformation genes on the array, 224 of these (86.5%) met our experiment-wide hybridization

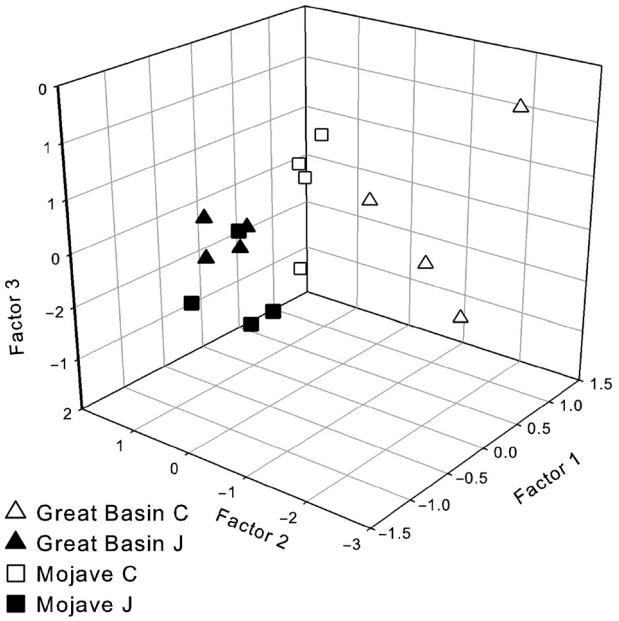

Fig. 1 The expression pattern of biotransformation genes of 16 *Neotoma lepida* represented as a function of the three first factors obtained in a PCA. PCA was based 178 probes with an intensity of 1 for all 16 woodrats. Spots with a quality lower than 1 were removed. Factors 1, 2, and 3 accounted for 47.9% of the total variance. Great Basin woodrats are indicated by triangles and Mojave by squares. Juniper diet (J) is indicated with solid symbols and creosote (C) with open.

standards for inclusion in the analysis. However, the number of spots included in each comparison may be less than 224 in order to meet similar quality control standards for each treatment included in a particular analysis.

The effect of diet on gene expression

There was a significant effect of diet on the expression of biotransformation genes across the two populations. Overall, gene expression was much more variable on the creosote diet than the juniper diet based on the greater level of dispersion of the data for the creosote diet (Fig. 1). The first seven factors of the PCA accounted for 73% of the total variance. No single gene contributed significantly to these factors.

Different sets of biotransformation genes were up-regulated on the creosote and juniper diets (Table 2a–c). Several of these genes showed a similar pattern of expression in both populations (Table 2a). Woodrat populations feeding on juniper had greater similarity in gene expression patterns than on creosote. The two populations of woodrats shared 49% of the up-regulated genes on juniper compared to 21% of those up-regulated on creosote.

The two woodrat populations also expressed unique sets of biotransformation genes. Within the Great Basin population alone, 20% of the biotransformation genes (41 of 208) were differentially expressed on creosote vs. juniper (Table 2a, b). Of these, 27 were expressed to a greater extent on juniper, whereas 14 were up-regulated on creosote (Table 2a, b). Within the Mojave woodrats, 26% of all biotransformation genes (56 of 211) were differentially expressed on juniper vs. creosote (Table 2a, c). Thirty of these were up-regulated on creosote whereas 26 were up-regulated on juniper.

Comparative biotransformation of creosote

Mojave and Great Basin woodrats ingesting a diet with creosote resin differed in gene expression of biotransformation enzymes. On the creosote diet, 14% (26/265) of the hybridized biotransformation genes on the array were significantly differentially expressed between Mojave and Great Basin woodrats (Table 3). Overall, the Mojave woodrats had greater expression of more biotransformation genes than the Great Basin woodrats on the creosote diet. Mojave woodrats expressed 24 genes to a greater extent than Great Basin woodrats (Table 3). These genes included four different cytochrome P450 (CYP) isozymes as well as genes representing five classes of conjugation pathways. In contrast, Great Basin woodrats feeding on creosote expressed only two genes to a greater extent than Mojave. These genes included one functionalization enzyme and one conjugation enzyme.

Comparative biotransformation of juniper

There was a similar pattern of expression between the two populations fed juniper with the exception that the Great Basin woodrats exhibited greater expression of more biotransformation genes than the Mojave woodrats. A total of 14% of the hybridized biotransformation genes on the array (30/222 genes) were differentially expressed between the two populations (Table 4). Great Basin woodrats expressed 21 genes to a greater extent than did Mojave woodrats. These included five CYP isozymes, a number of other functionalization enzymes and four classes of conjugation enzymes. The Mojave woodrats had greater expression of nine biotransformation enzymes compared to the Great Basin woodrats (Table 4). These included two CYPs and three enzymes related to the glutathione S-transferase conjugations pathways ('GST').

Quantitative PCR verification

The qPCR results for SOD, CYP2A3A and POR corroborated the expression profiles from the microarray. For each gene, we chose a specific diet-by-population comparison that passed the quality control and significance criteria of the microarray. For SOD and CYP2A3A, we compared the

Table 2 The effect of diet treatment on gene expression in Mojave and Great Basin woodrat populations. Data are divided by gene expression patterns that are shared by Mojave and Great Basin populations (2a), that are unique to Great Basin populations (2b) and that are unique to Mojave populations (2c). Gene ID number is the GenBank Accession number. The fold difference is expressed as the Log_2 ratio. *P* values were determined using a *T* test. CYP stands for cytochrome P450

(a) Genes that both populations of woodrats expressed in a similar manner on the different diet treatments (e.g. greater expression on creosote versus juniper)

		Mojave Desert		Great Basin	
Gene ID	Gene name	Log_2 ratio	*P* value	Log_2 ratio	*P* value
	Greater expression on juniper				
NM_013105	CYP3A3	3.25	0.00017	2.65	0.0011
XM_001070774	CYP2b15	2.98	0.00105	3.67	0.0018
NM_138515	CYP2d22	2.90	0.00255	2.19	0.0073
NM_173304	CYP2d10	2.66	0.00013	1.73	0.0417
NM_198733	CYP2b13	2.65	0.00035	3.03	0.0052
NM_031576	P450 (cytochrome) oxidoreductase	2.39	0.00042	1.93	0.0196
XM_001057230	Similar to glucosamine 6-phosphate N-acetyltransferase	2.31	0.00099	1.56	0.0130
NM_153312	CYP3a11	2.29	0.00054	2.29	0.0006
XM_341808	CYP2b2	2.25	0.00005	4.74	0.0003
XM_577774	Similar CYP 2b12	2.18	0.00106	2.97	0.0012
NM_020540	Glutathione S-transferase M4	2.10	0.00514	1.35	0.0269
XM_001062874	CYP2b12	2.08	0.00071	2.06	0.0057
NM_012844	Epoxide hydrolase 1, microsomal	2.03	0.00110	1.87	0.0132
NM_201423	UDP glycosyltransferase 1A6	1.91	0.00004	1.48	0.0406
NM_053906	Glutathione reductase	1.80	0.00269	1.79	0.0006
NM_001007602	Glutathione S-transferase omega 1	1.78	0.00067	1.73	0.0077
NM_017158	CYP2c7	1.74	0.00570	4.97	0.0003
NM_145782	CYP3a18	1.54	0.01229	1.73	0.0002
NM_173294	CYP2b3	1.43	0.03583	1.75	0.0310
	Greater expression on creosote				
NM_012792	Flavin containing monooxygenase 1	3.84	0.00060	2.24	0.0201
NM_001025423	Alcohol dehydrogenase, iron containing, 1	2.26	0.00007	1.73	0.0004
NM_030826	Glutathione peroxidase 1	1.91	0.00022	1.42	0.0022
NM_031329	Thiopurine methyltransferase	1.65	0.02538	1.87	0.0114
NM_001011975	Aldehyde dehydrogenase 1B1	1.61	0.01815	1.69	0.0246
NM_031543	CYP2e1	1.55	0.00075	1.53	0.0013
AI234527	Glutathione S-transferase, alpha 4	1.47	0.03933	1.69	0.0005
BQ191682	Glutathione S-transferase, mu 5	1.33	0.00403	2.08	0.0322

(b) Genes that were differentially expressed in only Great Basin woodrats on juniper versus creosote diets

Gene ID	Gene name	Log_2 ratio	*P* value
	Greater expression on juniper		
BQ199447	Aldo-keto reductase 1C12	2.34	0.0492
XM_001064425	Carboxylesterase 1	2.26	0.0344
AA925792	Superoxide dismutase 1	1.54	0.0005
AY325187	Acetyl-Coenzyme A acetyltransferase 2	1.49	0.0072
XM_001067959	Similar to Catechol O-methyltransferase (predicted)	1.30	0.0237
NM_144737	Flavin containing monooxygenase 2*	1.26	0.0134
AW914895	Aldo-keto reductase 1B10 (aldose reductase)	1.25	0.0193
NM_022273	Aldehyde dehydrogenase 9A1	1.24	0.0273
	Greater expression on creosote		
NM_177426	Glutathione S-transferase, mu 2	2.57	0.0007
NM_001007667	Spermidine/spermine N1-acetyl transferase	1.55	0.0002
XM_214712	Sulfotransferase 5A1 (predicted)	1.50	0.0164
NM_013198	Monoamine oxidase B	1.48	0.0057
XM_214535	Aldehyde dehydrogenase 7A1	1.37	0.0133
XM_576003	Similar to alcohol dehydrogenase PAN2 (predicted)	1.15	0.0254

Table 2 *Continued*
(c) Genes that were differentially expressed in only Mojave woodrats on juniper versus creosote diets

Gene ID	Gene name	Log_2 ratio	*P* value
	Greater expression on juniper		
NM_019170	Carbonyl reductase 1	3.05	0.0007
XM_221641	Carbonyl reductase 3 (predicted)	1.91	0.0188
XM_217906	CYP2c55 (predicted)	1.49	0.0259
NM_012683	UDP glycosyltransferase 1A6	1.35	0.0059
XM_217138	Nicotinamide N-methyltransferase (predicted)	1.29	0.0479
XM_347254	Similar to Putative methyltransferase WBSCR22	1.24	0.0416
BQ196649	Glutathione peroxidase 2	1.23	0.0095
	Greater expression on creosote		
NM_172038	Glutathione S-transferase, mu 5	2.76	0.0324
XM_215682	Glutathione S-transferase, mu 6 (predicted)	2.46	0.0028
XM_213943	Microsomal GST 3 (predicted)	2.39	0.0001
NM_031834	Sulfotransferase 1A1, phenol-preferring	2.36	0.0027
NM_012542	CYP2A3a	2.02	0.0053
M33313	CYP2A1	1.89	0.0397
NM_012796	Glutathione S-transferase, theta 2	1.86	0.0022
AA819129	Similar to Glutathione S-transferase, theta 3 (predicted)	1.84	0.0429
BF288683	Alcohol dehydrogenase, iron containing, 1	1.76	0.0369
NM_147206	CYP3a13	1.68	0.0337
XM_343764	Monoamine oxidase A	1.62	0.0143
NM_053425	Copper chaperone for superoxide dismutase	1.60	0.0026
XM_574740	Similar to Glutathione S-transferase, theta 3 (predicted)	1.44	0.0170
NM_032416	Aldehyde dehydrogenase 2	1.42	0.0074
NM_012541	CYP1a2	1.41	0.0483
XM_214478	Aldehyde dehydrogenase 5A1	1.38	0.0411
AI407458	Aldehyde dehydrogenase 6A1	1.37	0.0210
NM_017014	Glutathione S-transferase, mu 1	1.33	0.0465
AA875107	Similar to NADH-ubiquinone oxidoreductase B9	1.31	0.0050
XM_340825	Spermidine/spermine N1-acetyl transferase 2 (predicted)	1.28	0.0059
NM_017050	Superoxide dismutase 1	1.22	0.0059
NM_144737	Flavin containing monooxygenase 2	1.16	0.0396

expression levels of Mojave and Great Basin populations on the creosote diet. Both genes were more highly expressed in the Mojave animals (SOD: 2.9-fold average, CYP2A3A: 4.2-fold average), consistent with the microarray (Table 3). POR was compared across the Great Basin populations fed either juniper or creosote, and as in the microarray (Table 2a), greater expression was found in the juniper diet treatments (1.5-fold average).

Discussion

Identifying the key biotransformation mechanisms that herbivores use when ingesting plant secondary compounds is crucial for a thorough understanding of plant–mammal interactions. Studies on the biotransformation mechanisms of wild herbivores have been previously hindered by the lack of reagents and assays available for nonmodel systems. We overcame this limitation by using microarrays designed for laboratory rats to explore three hypotheses. First, we tested the hypothesis that plants with different secondary compound profiles are metabolized by different sets of biotransformation enzymes. Next, we tested the hypothesis that woodrats in the Mojave Desert use a different set of biotransformation enzymes for metabolizing creosote than a population of conspecifics naïve to creosote. Lastly, we tested whether Mojave woodrats biotransform secondary compounds in their putative ancestral diet of juniper using enzymes comparable to those used by a population that currently feeds on juniper. Overall, we found large differences in the enzymes used to biotransform secondary compounds in juniper vs. creosote. Furthermore, the results are consistent with the hypothesis that Mojave woodrats have evolved a unique strategy for the biotransformation of creosote resin. Although the Mojave population appears to retain the ability to ingest similar quantities of juniper compared to the Great Basin population, notable differences existed with respect to expression of biotransformation genes between the two populations when consuming

Table 3 Differential gene expression in Great Basin versus Mojave woodrats on the creosote diet treatment. Gene ID number is the GenBank Accession number. The fold difference is expressed as the Log_2 ratio. *P* values were determined using a T test. CYP stands for cytochrome P450

Gene ID	Gene name	Log_2 ratio	*P* value
	Greater expression in Great Basin woodrats		
NM_019170	Carbonyl reductase 1	2.17	0.02992
XM_214712	Sulfotransferase 5A1 (predicted)	1.45	0.04694
	Greater expression in Mojave woodrats		
BQ199447	Aldo-keto reductase 1C12	2.66	0.04987
XM_001064425	Carboxylesterase 1	2.12	0.04563
NM_022228	UDP glucuronosyltransferase 2A1	2.03	0.03920
NM_012730	CYP2d26	2.01	0.029
AA925792	Superoxide dismutase 1	1.90	0.00013
NM_012542	CYP2A3a	1.82	0.00027
NM_147206	CYP3a13	1.82	0.04008
XM_225544	Similar to aldo-keto reductase 1C12 (predicted)	1.73	0.03046
XM_213943	Microsomal GST 3 (predicted)	1.52	0.01929
NM_017050	Superoxide dismutase 1	1.52	0.03464
NM_017013	Glutathione S-transferase, alpha type 2	1.48	0.02663
NM_053425	Copper chaperone for superoxide dismutase	1.40	0.01127
NM_022273	Aldehyde dehydrogenase 9A1	1.39	0.04671
NM_012796	Glutathione S-transferase, theta 2	1.38	0.02955
XM_340825	Spermidine/spermine N1-acetyl transferase 2 (predicted)	1.30	0.00945
NM_031834	Sulfotransferase 1A1, phenol-preferring	1.30	0.03303
AW914895	Aldo-keto reductase 1B10 (aldose reductase)	1.28	0.01260
AI407458	Aldehyde dehydrogenase 6A1	1.27	0.00094
NM_144737	Flavin containing monooxygenase 2	1.25	0.02684
M33747	UDP glucuronosyltransferase 2, member 5	1.24	0.04518
XM_001067959	Similar to Catechol O-methyltransferase (predicted)	1.22	0.01803
XM_341808	CYP2b2	1.21	0.02455
AW251950	Catechol-O-methyltransferase domain containing 1 (predicted)	1.14	0.02392
XM_214526	Zinc-binding alcohol dehydrogenase 2 (predicted)	1.10	0.04623

juniper. Thus, within the Mojave population that no longer feeds on juniper, the genes for biotransformation of juniper may no longer be constrained by strong selective pressure to feed on juniper. In the subsequent paragraphs, we elaborate on these results as well as discuss the use of microarrays for nonmodel systems.

The effect of diet on patterns of biotransformation enzymes

Switching from one diet to a novel one with a different nutritional background represents a considerable challenge for herbivores. The plants used for the diet treatments in this study had notable differences in their secondary compound profiles. Polyphenolics (aromatic rings with hydroxyl groups) constitute the largest class of secondary compounds in creosote. The main component of resin is nordihydroguaiaretic acid (NDGA), a lignan catechol (Mabry *et al*. 1977). In contrast, terpenes (polymers of C5 hydrocarbons) are the primary class of secondary compounds in juniper (Schwartz *et al*. 1980; Adams *et al*. 1981). The differences in the chemical structures of the compounds in juniper and creosote imply that they would be processed at least in part by different sets of biotransformation enzymes (Klaassen 2001).

The work presented herein is the first to examine on a large scale (i.e. > 200 enzymes), the differential expression of biotransformation enzymes in mammalian herbivores feeding on plant species that vary in secondary compound profiles. The results support the contention that disparate classes of secondary compounds are processed by different sets of biotransformation enzymes. The pattern of gene expression in both populations feeding on juniper compared to creosote suggests that woodrats may rely heavily on functionalization enzymes in the biotransformation of juniper. In particular, CYP isozymes appear key in the metabolism of juniper as both populations had elevated expression of 13 CYP isozymes on juniper compared to creosote treatments (Table 2a). All of the elevated isozymes are from CYP families (1–3) with documented importance in drug metabolism (Martignoni *et al*. 2006). Moreover, of all the differentially expressed enzymes, the CYP isozymes ranked in the top five exhibiting the greatest fold change

Table 4 Differential gene expression in Great Basin versus Mojave woodrats on the juniper diet treatment. Gene ID number is the GenBank Accession number. The fold difference is expressed as the Log_2 ratio. *P* values were determined using a *T* test. CYP stands for Cytochrome P450

Gene ID	Gene name	Log_2 ratio	*P* value
	Greater expression in Great Basin woodrats		
NM_031329	Thiopurine methyltransferase	2.58	0.004
NM_172038	Glutathione S-transferase, mu 5	2.43	0.021
XM_574039	Glutathione peroxidase 5	2.23	0.019
XM_213943	Microsomal GST 3 (predicted)	1.64	0.003
NM_001013084	Aldo-keto reductase 1B10 (aldose reductase)	1.63	0.043
NM_017050	Superoxide dismutase 1	1.45	0.003
NM_001008522	Aldehyde oxidase 2	1.39	0.010
AA858639	Catechol-O-methyltransferase	1.39	0.031
XM_001063361	CYP2c37	1.35	0.018
BM986667	Epoxide hydrolase 2, cytoplasmic	1.35	0.040
XM_219933	CYP2c65 (predicted)	1.30	0.045
NM_001011975	Aldehyde dehydrogenase 1B1	1.27	0.030
NM_173323	UDP glucuronosyltransferase	1.23	0.019
NM_057105	UDP glycosyltransferase 1A6	1.22	0.010
NM_017156	CYP2b15	1.19	0.001
NM_012940	CYP1b1	1.18	0.014
NM_144743	Carboxylesterase 6	1.18	0.028
NM_030826	Glutathione peroxidase 1	1.17	0.001
NM_001025423	Alcohol dehydrogenase, iron containing, 1	1.17	0.050
NM_144737	Flavin containing monooxygenase 2	1.16	0.008
XM_341808	CYP2b2	1.14	0.021
	Greater expression in Mojave woodrats		
AI029806	Superoxide dismutase 2, mitochondrial	2.03	0.01377
NM_012730	CYP2d26	2.04	0.025
NM_173304	CYP2d10	1.90	0.00001
NM_177426	Glutathione S-transferase, mu 2	1.90	0.00146
NM_020540	Glutathione S-transferase M4	1.80	0.00010
NM_019170	Carbonyl reductase 1	1.76	0.04828
NM_001009920	Glutathione S-transferase Yc2	1.48	0.04559
XM_574740	Similar to glutathione S-transferase, theta 3 (predicted)	1.42	0.03190
XM_001057230	Similar to glucosamine 6-phosphate N-acetyltransferase	1.28	0.046

(6× or more). In other mammalian systems, CYPs have been documented to biotransform select terpenes (Pass *et al*. 1999, 2002). In contrast, for both populations on creosote, multiple enzymes in the glutathione conjugation pathway ('GSTs') were up-regulated compared to a juniper diet. The Mojave population fed with creosote had elevated expression of an additional seven probes for enzymes related to the glutathione pathway (Table 2c). Polyaromatic hydrocarbons such as those present in creosote resin are known substrates of the glutathione pathway (Klaassen 2001). Overall, the results support the hypothesis that different PSCs are metabolized by different biotransformation enzymes.

Previous studies on the urinary metabolites of mammals feeding on natural compounds provide initial support for the idea that PSCs with different chemical structures are processed to a certain extent by distinct sets of biotransformation enzymes. Two species of possums fed two species of *Eucalyptus* produced urines that differed in pH between diets (Foley 1992; Dearing & Cork 1999; Wiggins *et al*. 2003). One interpretation of this result is that the differences in urinary pH stem from alternative processing of the PSCs in each *Eucalyptus* species such that the urinary metabolites differ in acidity. The PSC profiles of creosote and juniper are likely to be far more different from each other than the PSCs of these two Eucalypt species. In contrast, other studies have documented overlap among biotransformation pathways in the processing of different PSCs (Wiggins *et al*. 2003; Marsh *et al*. 2006). This result is consistent with the broad substrate acceptability of many biotransformation enzymes (Klaassen 2001). A limitation of these studies in addressing the initial hypothesis is that urinary metabolites cannot often be linked to individual biotransformation enzymes, per se, but are restricted to association with a

particular pathway containing multiple enzymes (e.g. the variety of UDP-glycosyltransferases in the glucuronidation pathway (Bock 2003)). We remedied this problem by using microarray technology, which enables us to pinpoint specific biotransformation genes that are correlated with the metabolism of these different PSCs.

We acknowledge that the results are based on gene expression, which does not always translate into differences in protein quantity and also that the large number of comparisons generated by a microarray study yields some fraction of results that are false discoveries. Thus, not all of the genes expressed to a greater extent on a particular diet maybe those that metabolize the secondary compounds in a species. Nonetheless, the data permit an initial investigation into the putative biotransformation enzymes and general patterns of metabolism.

Mechanisms for eating creosote

Approximately 18 700 years ago, populations of *Neotoma lepida* in the southwestern USA and northern Baja California, Mexico experienced a radical change in flora as a result of a natural climatic event. Juniper trees were replaced by creosote bush thereby presenting a novel dietary challenge for the herbivores in that area. To begin to identify the potential candidate biotransformation genes in Mojave woodrats that have resulted in their ability to rely on creosote, we compared gene expression of Mojave woodrats consuming creosote to a closely related woodrat population with no previous experience with creosote. The two populations used in this study are from the same phylogenetic subclade (2A) and share a common ancestor within the past 60 000 years (Patton *et al*. 2008). The genetic diversity within this subclade overall is low with the majority of haplotypes present across multiple localities.

Despite the low genetic diversity across these populations, there were differences in the patterns of expression of biotransformation genes between Mojave and Great Basin woodrats on the creosote treatment. Mojave woodrats had greater expression of 10× as many biotransformation genes as did the Great Basin woodrats consuming a diet of creosote resin (Table 3). Moreover, the types of biotransformation genes expressed to a greater degree in Mojave woodrats are ones known to metabolize polyphenolic compounds, and thus, could be key in the biotransformation of a diet containing creosote resin. Mojave woodrats had greater expression levels of three mono-oxygenases (CYP2A3, CYP3A13 and one flavin) compared to the Great Basin woodrats where no mono-oxygenases were up-regulated. CYP2A metabolizes numerous xenobiotics (Pearce *et al*. 1992; Pasanen & Pelkonen 1994; Longo *et al*. 2004). CYP2A3 in particular, metabolizes coumarin, a phenolic PSC (Honkakoski *et al*. 1993). Less has been reported with respect to CYP3A13; however, the subfamily CYP3A is critical for metabolism of drugs in humans. This subfamily alone metabolizes more than 50% of all drugs. Preliminary studies on CYP3A13 are indicative of drug-metabolizing properties. In addition, expression levels of superoxide dismutase (SOD) and its chaperone were also greater in Mojave woodrats. Although SOD is not a biotransformation enzyme *sensu stricto*, its ability to scavenge free radicals could be important in protecting cells from oxidative damage caused either directly from PSCs in creosote resin or from biotransformation metabolites (McCord & Edeas 2005). Thirty-eight per cent (9/24) of the elevated transcripts in Mojave woodrats were related to several conjugation pathways including catechol-o-methyltransferase, N-acetylation, sulphation, glutathione and glucuronidation. Catechol-o-methyltransferase (COMT) acts on phenolic substrates and has been implicated as playing a key role in dietary specialization of different species of *Neotoma* (Skopec *et al*. 2007). Thus, it is possible that COMT biotransforms creosote resin. However, it should be noted that the results for COMT were based on probes 'similar to' or 'predicted' as COMT and not ones designed from COMT sequence. Therefore, the microarray results may not represent differential expression of COMT transcripts. Other studies have suggested that glutathione and glucuronidation, in particular, may be central in the metabolism of PSCs by woodrats (Mangione *et al*. 2004; Haley *et al*. 2008). Elevated expression of UDP glucuronosyltransferase enzymes is consistent with a previous study that found Mojave woodrats fed with creosote excreted greater levels of urinary glucuronides compared to Great Basin woodrats (Mangione *et al*. 2001).

In contrast, Great Basin woodrats on the creosote treatment had elevated expression of only two genes, one functionalization enzyme and one conjugation enzyme (Table 3). Sulfotransferase is part of the sulphation conjugation pathway. This pathway can act on many substrates; however, sulphation is generally considered an auxiliary pathway because the conjugate is often in limited supply (Klaassen 2001). The potential elevated use of sulphation by Great Basin woodrats may be the result of not having other pathways to biotransform the novel toxin creosote. The extent of the differences in gene expression between the Mojave and Great Basin populations yield a reasonable number of candidates to screen for future functional assays to determine which enzymes are central to the metabolism of creosote.

Concordance of results with enzyme activity assays

Microarray results should be interpreted cautiously with respect to function, given that transcripts can be alternatively spliced, which may ultimately result in different functions (Derome *et al*. 2006). Moreover, transcript levels do not necessarily reflect the cellular levels of proteins or enzyme

activities (Du *et al*. 2004). However, the results of a previous study on the CYP content and enzyme activity of a subset of seven specific biotransformation pathways of Mojave and Great Basin woodrats are consistent in many ways with the microarray results. Activity levels of glutathione-s-transferase, CYP2B, and total CYP content were greater in Mojave woodrats compared to Great Basin woodrats fed with creosote (Haley *et al*. 2008). These enzyme activity results correspond to the greater levels of gene expression in the microarray experiment. Furthermore, in the microarray experiment, the Mojave woodrats had elevated expression of three CYPs compared to Great Basin woodrats. Greater expression of these CYPs by Mojave woodrats could produce a difference in total CYP content as documented by Haley *et al*. (2008). In addition, the Great Basin woodrats had greater enzyme activity of sulfotransferase compared to Mojave woodrats. This difference in activity could be the result of elevated transcription of sulfotransferase 5A1 in the Great Basin woodrats, as suggested by our microarray results. Lastly, Haley *et al*. (2008) documented no difference in quinone oxido-reductase activity, which is consistent with the microarray results.

There were inconsistencies between the microarray expression assay and the enzyme activity assays. The activity of two enzymes (UDP-glucuronosyltransferases and CYP3A) from the Haley *et al*. (2008) study did not match the expression patterns observed in the microarrays in that there was no difference in enzyme activity but there were differences in expression for corresponding enzymes on the microarrays. There was no difference in activity of UDP-glucuronosyltransferases, key enzymes in the glucuronidation pathway. However, in the microarray experiment, Mojave woodrats had greater expression of two glucuronosyltransferases (UGP2-5 and UGP2A1) compared to the Great Basin creosote-fed woodrats. The results of a separate study, which took a whole organism approach, found increased glucuronidation capacities of Mojave woodrats (Mangione *et al*. 2001). In that study, Mojave woodrats had 2× greater excretion rates of urinary glucuronic acid compared to Great Basin woodrats fed resin diets (Mangione *et al*. 2001). CYP3A enzyme activity exhibited a similar pattern of discordance in that activity levels did not differ but expression of CYP3A13 was greater in Mojave woodrats. There are no comparable whole organism studies in woodrats for CYP3A metabolism.

There are at least three possible explanations for the disparity between the enzyme activity assays and the microarray results for UDP-glucuronosyltransferases and CYP3A. First, it is possible that the differences in expression are false positives in the microarray. Second, the substrates used in the *in vitro* activity assay may not be substrates of the up-regulated enzymes on the microarray, and thus, no difference in activity would be present regardless of protein content differences. This explanation seems most plausible for the glucuronidation pathway given the results of the whole organism study. Lastly, the enhanced expression of the up-regulated transcripts may not have resulted in increased protein content (i.e. if the transcripts represented pseudogenes). Distinguishing between these alternatives requires further study.

The reverse incongruity between the activity studies and the microarray experiment occurred for a single enzyme. Mojave woodrats had higher induced activity of CYP1A (Haley *et al*. 2008), yet there was no apparent difference in expression between the populations. This was not the result of inadequate binding of woodrat cDNA to the rat probes on the microarray as hybridization to CYP1A probes met quality control requirements indicating adequate binding of sample. The lack of differential expression combined with a difference in activity suggests that there may be functional differences in this enzyme that lead to greater activity without greater expression. This hypothesis warrants additional investigation.

Biotransformation on a novel diet vs. putative ancestral diet

Herbivores feeding on their natural diet appear to have more options for processing the PSCs with which they have evolutionary experience. Mojave woodrats fed with creosote had elevated expression of more than 10× as many transcripts as the Great Basin woodrats. Similarly, the Great Basin woodrats fed juniper had nearly 3× the number of elevated transcripts compared to the Mojave woodrats. Further support for this interpretation is that the Mojave woodrats have a greater response to juniper with which they have had recent evolutionary experience compared to Great Basin woodrats feeding on a completely novel plant, creosote. However, both populations lost mass on the juniper diet; thus, Great Basin animals may not be more efficient at metabolizing juniper than the Mojave animals despite the increased number of transcripts. These results also imply that dietary shifts between plants with disparate chemistry such as juniper and creosote may necessitate more extensive changes in biotransformation mechanisms than plants with similar chemistries such as juniper and cedar (both terpene-rich). Furthermore, little is known about the effect of exposure during development on the biotransformation capacity of woodrats. All the animals in this study were captured as adults in the wild; thus, we cannot address differences in developmental acclimation or preference. Preliminary work on a related species of woodrat (*Neotoma stephensi*) suggests that animals raised from birth on rabbit chow (including the maternal diet during lactation) are equally capable of consuming juniper as wild-caught adults (A-M. Torregrossa, personal communication). However, even this work does not address conditions during gestation. Clearly, more investigation is

necessary to fully address these concepts. For example, it would be interesting to determine whether the populations of *N. lepida* that were first exposed to creosote followed its expansion northward, thereby displacing or replacing previous populations of woodrats or whether the ability to biotransform creosote evolved *in situ* repeatedly.

Use of microarrays on nonmodel systems

The development of microarray technology presents an unprecedented opportunity to examine the expression of thousands of genes simultaneously. This technology has applications to many fields and disciplines. One concern has been its applicability to species other than that for which the array was designed. In our study using *Neotoma* samples, we had excellent hybridization to a microarray designed for rats. We had acceptable hybridization to more than 70% of the probes on the array. This is considerably greater than previous cross-species studies (Moody *et al*. 2002). One possible explanation for the greater hybridization in this study was that our target animals were more closely related, i.e. in the same family, to those for which the chip was designed compared to the other studies where comparisons were across families or orders. The next decade will bring a tremendous increase in the availability of microarrays designed for an even greater number of species. Moreover, new pyrosequencing techniques combined with the availability of custom oligo-microarrays will permit the design of microarrays for nearly any nonmodel system for which a transcriptome can be described. These new technologies will greatly facilitate ecological and evolutionary studies on nonmodel systems.

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This project was part of Elodie Magnanou's research as a postdoctoral fellow at the University of Utah. She is now a faculty member in the Laboratoire Arago (UPMC Univ Paris 06—CNRS). She combines functional genomics tools, genetic markers and physiology to address questions related to the ecology, and the genetic basis of adaptation in natural populations. J. R. Malenke is interested in the ecological and evolutionary factors governing intimate interspecific interactions. As a graduate student, she worked on host-parasite relationships using a bird/ectoparasitic louse system. She is currently investigating the genetic diversity of woodrat detoxification pathways in the Dearing lab. M. D. Dearing has worked on the nutritional ecology of vertebrate herbivores since 1985 and on woodrats in particular since 1996. Her current research focuses on understanding the mechanisms of biotransformation in woodrat herbivores as well as the response of woodrat herbivores to climate change induced increases in ambient temperature.
