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Differential hepatic gene expression of a dietary specialist (*Neotoma stephensi*) and generalist (*Neotoma albigula*) in response to juniper (*Juniperus monosperma*) ingestion

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Abstract

Dietary specialization is thought to be rare in mammalian herbivores because of limitations of their detoxification system in processing large doses of a single type of plant secondary compound (PSC). Therefore, in order to specialize on a single species of plant, mammalian herbivores must have a highly efficient detoxification system for the particular types of PSCs they ingest. Using microarray technology, we looked at the expression of hepatic genes of a dietary specialist, *Neotoma stephensi*, and a sympatric generalist, *Neotoma albigula*, in response to diets containing different levels of one-seeded juniper (*Juniperus monosperma*). We found large between species differences in gene expression, as well as large within species differences when specialists fed a low juniper diet (25% juniper) were compared to specialists fed their ecologically relevant level of juniper (70% juniper). We also tested the hypothesis that the specialist relies on less costly phase I detoxification enzymes more than phase II compared to the generalist. Although we found that the specialist had higher cumulative as well as average expression of phase I versus phase II enzymes, the generalist had a similar pattern of expression for phase I versus phase II enzymes.
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1. Introduction

Dietary specialization is uncommon in mammalian herbivores; it is estimated that less than one percent of mammalian herbivores sustain themselves on a single species of plant (Freeland, 1991). Freeland and Janzen (1974) proposed that the lack of dietary specialization in mammalian herbivores is due to a limitation of the detoxification system in processing large quantities of a single type of plant secondary compound (PSC). It is thought that most mammalian herbivores eat a variety of plants to avoid consuming high levels of a single PSC that would overwhelm their detoxification system. When Freeland and Janzen proposed their theory more than 30 years ago, little was known about the detoxification system of wild herbivores. Recently several advances have resulted from the incorporation of pharmacological and toxicological techniques to the field of plant mammal interactions. Thus, far more is known about the detoxification systems of wild herbivores (Sorensen et al., 2006). This progress resulted in a revision of Freeland and Janzen's theory such that specialists are predicted to rely more on phase I enzymes that are less energetically costly than phase II enzymes (McLean et al., 1993, 2001 McLean and Foley, 1997; Boyle et al., 2001).

The mammalian detoxification system is separated into two phases. Phase I or functionalization enzymes hydrolyze, reduce or oxidize compounds by adding or exposing a functional group (hydroxyl, amine, thiol or carboxyl) to the compound. The functionalized compound can either be excreted into the urine or bile, or enter phase II for conjugation. Phase II or conjugation enzymes, place a conjugate (an amino acid, carbohydrate, sulfate, acetyl or methyl group) onto the compound being metabolized, making it more water soluble for excretion in urine

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or bile. Phase I enzymes are usually more substrate specific but sometimes produce metabolites that are more reactive than the parent compound, thereby enhancing toxicity (Morse and Stoner, 1993; Ayala and Cutler, 1997). Phase II enzymes in contrast, have broader substrate acceptability. They tend to produce less reactive metabolites and are therefore thought as "safer" pathways (Foley and McArthur, 1994; Parkinson, 1996). However, phase II enzymes utilize more energy during detoxification because the conjugate is lost when the metabolite is excreted. It is hypothesized that specialist mammalian herbivores rely less on phase II enzymes to minimize the loss of conjugates than generalists (McLean et al., 1993, 2001; McLean and Foley, 1997; Boyle et al., 2001). Because specialists theoretically consume fewer types of PSCs, they can rely more on the substrate specific pathways of the phase I system as long as the metabolites formed are not more toxic than the parent compound. Therefore, dietary specialists are predicted to have higher levels of phase I enzymes to detoxify the high dose of PSCs in the most energetically efficient manner possible. Supporting evidence for this hypothesis is dietary generalists excrete higher levels of conjugated metabolites in urine (i.e., processed via phase II enzymes) compared with dietary specialists (McLean and Foley, 1997; Boyle et al., 1999, 2001; Sorensen et al., 2005).

We have been studying dietary specialization within the woodrats, genus *Neotoma*. Three different species in the genus are dietary specialists on three different plants with very disparate PSCs. In this paper, we focus on one specialist *N. stephensi* and its sympatric generalist, *N. albigula*. *Neotoma stephensi* is a specialist on *Juniperus monosperma* (one-seeded juniper), which comprises 70% or more of its diet. *Neotoma albigula*, in contrast, consumes only 25% juniper in its diet (Vaughan, 1982; Dial, 1988). Juniper is an evergreen containing high levels of monoterpenes, with alpha-pinene being the most abundant terpene (2% dry weight, Adams et al., 1981; Dearing et al., 2000). The toxic action of alpha-pinene in mammals is central nervous system depression, contact dermatitis, lung function impairment, liver and kidney cysts and death (Sperling et al., 1967; Savolainen, 1978; Falk et al., 1990).

Previous studies report that the differential capacities for juniper consumption of *N. stephensi* and *N. albigula* are not due to nutritional factors such as a greater ability to maintain nitrogen balance (McLister et al., 2004; Dearing et al., 2005, in press; Sorensen et al., 2005). Instead, it seems that *N. stephensi* has a superior ability to detoxify alpha-pinene and other terpenes in juniper (Sorensen and Dearing, 2003; Sorensen et al., 2004). The activities of a handful of detoxification enzymes revealed that *N. stephensi* and *N. albigula* have disparate levels of activities for phase I versus phase II enzymes when fed terpene-free diets as well as juniper diets (Haley et al., in review). We wished to expand on these data by simultaneously measuring the expression of thousands of genes in the liver using microarray technology. We examined whether the specialist's greater capacity for juniper consumption is due to unique hepatic gene regulation in response to juniper or innate differences between the species in hepatic gene expression. We also used the data to evaluate the hypothesis that specialists utilize phase I pathways to a greater extent than phase II.

2. Materials and methods

2.1. Woodrats

Neotoma stephensi and *N. albigula* were trapped near Wupatki National Park, 45 km northeast of Flagstaff, Arizona, USA (35°30′ N, 111°27′ W) and transported to the University of Utah Department of Biology's Animal Facility. Woodrats were housed in individual cages (48 × 27 × 20 cm) with pine shavings. Environmental conditions were 12:12-h light:dark cycle, ambient temperature of 28 °C and humidity of 15% for at least 6 months prior to experiments. The woodrats were fed standard rabbit chow (Harlan Teklad formula 2031) and water *ad libitum*. All animals were screened for hantavirus prior to experimentation. All experimental procedures involving woodrats were approved by the University of Utah's Institutional Animal Care and Use Committee protocol number 01-02002.

2.2. Dietary treatments

To reduce variability, we used only males in feeding trials. Woodrats were fed three experimental diets (terpene-free, 25% juniper and 70% juniper). The terpene-free diet was ground rabbit chow (Harlan Teklad formula 2031). The 25% juniper diet was 75% ground rabbit chow and 25% ground juniper on a dry weight basis. The 70% juniper diet was 30% ground rabbit chow and 70% ground juniper on a dry weight basis. The juniper used in the dietary treatments (*Juniperus monosperma*) was collected from trees at woodrat trapping sites and kept frozen at −20 °C until use. The juniper was crushed on dry ice until it passed through a 1.0 mm screen. The diets were made daily to minimize volatilization of the terpenes.

Table 1
Experimental layout of microarrays

Array	Species	Diet	Dye
Between species			
1	*N. albigula*	Terpene-free	Red
	N. stephensi	Terpene-free	Green
2	*N. albigula*	Terpene-free	Green
	N. stephensi	Terpene-free	Red
3	*N. albigula*	25% juniper	Red
	N. stephensi	25% juniper	Green
4	*N. albigula*	25% juniper	Green
	N. stephensi	25% juniper	Red
Between treatments			
5	*N. albigula*	Terpene-free	Red
	N. albigula	25% juniper	Green
6	*N. albigula*	Terpene-free	Green
	N. albigula	25% juniper	Red
7	*N. stephensi*	Terpene-free	Red
	N. stephensi	25% juniper	Green
8	*N. stephensi*	Terpene-free	Green
	N. stephensi	25% juniper	Red
9	*N. stephensi*	25% juniper	Red
	N. stephensi	70% juniper	Green
10	*N. stephensi*	25% juniper	Green
	N. stephensi	70% juniper	Red

2.3. Experimental protocol

The woodrats were randomly assigned to three different treatment groups: control (terpene-free diet), juniper (25% juniper diet) and high juniper (70% juniper diet). The control group represented the non-induced group. The control group, consisting of two *N. stephensi* and two *N. albigula*, were fed the terpene-free diet for five days. The juniper group, consisting of two *N. stephensi* and two *N. albigula*, were fed an acclimation treatment of 5% juniper diet for two days and then the 25% juniper diet for three days. The 25% juniper diet represented the concentration of juniper that the *N. albigula* consumes in the field (Dial, 1988). The high juniper group consisted of only *N. stephensi*, because *N. albigula* cannot maintain body mass at this concentration. Two *N. stephensi* in the high juniper group were fed 5% juniper diet for one day, 25% juniper diet for two days and 70% juniper diet for three days. Food intakes were measured daily. At the end of the five day trials, the animals were euthanized with CO_2 and their livers were perfused with cold saline, harvested and stored at −80 °C.

To balance biological and technical replication with the expense of microarray experiments, a small sample size was used (Churchill, 2002). Two animals per group is a small sample size for statistical analysis. But for the mixed-model ANOVA used to compare each treatment, the residual degree of freedom is 5 (10 animals minus 5 treatments), which is considered acceptable in microarray experiments (Churchill, 2002).

2.4. RNA preparations

A 100 mg section of the livers were used for RNA extraction. Total RNA was extracted using TriZol reagent (Invitrogen) per the manufacturer's protocol and purified using an RNAeasy kit (Qiagen) as per manufacturer's protocol. The quality of the RNA was assessed using an RNA 6000 Nano Lab-on-a-Chip Kit with a 2100-Bioanalyzer system (Agilent Technologies) and quantity was

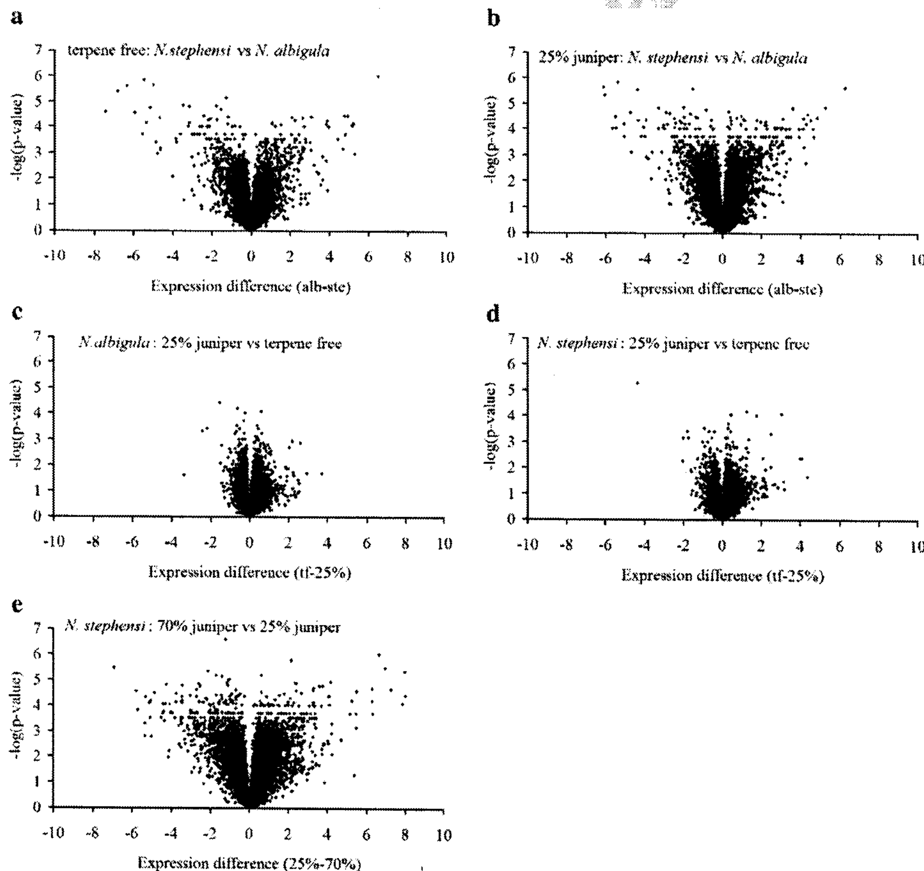

Fig. 1. a–e: Volcano plots of significance versus fold change in hepatic expression of genes in the dietary specialist *N. stephensi* and its sympatric generalist *N. albigula*. Each point represents a single gene analyzed by mixed-model ANOVA. A "typical" volcano plot exhibits the pattern of an erupting volcano with data points clustered into a small point at the center and bottom of the figure where genes that show low fold differences as well as low significance are clustered. The upward and outward data represent genes with larger fold differences and higher significance values. Expression difference is plotted as least-squares mean differences between (a) terpene-free diet: *N. stephensi* versus *N. albigula* (b) 25% juniper diet: *N. stephensi* versus *N. albigula* (c) *N. albigula*: 25% juniper versus *terpene*-free diet (d) *N. stephensi*: 25% juniper versus terpene-free diet (e) *N. stephensi*: 25% juniper versus 70% juniper.

determined spectrophotometrically. Total RNA (10 μg per sample) was reverse transcribed and labeled with fluorescent dyes to make fluorescently labeled cDNAs to be hybridized to the microarrays. The total RNA was reverse transcribed and labeled with Cyanine-3 CTP or Cyanine-5 CTP using Agilent technologies' Fluorescent Direct Label kit as specified by the manufacturer.

2.5. Microarray experiments

The fluorescently labeled cDNAs were hybridized to Agilent Technologies 60mer oligonucleotide rat microarrays per the manufacturer's instructions. More than 20,000 rat transcripts were present on the arrays, developed in conjunction with the National Institute of Environmental Health and Safety specifically for the field of toxicogenomics. A total of 10 microarrays were used with a semi-loop design to best determine inter as well as intra species effects (Table 1). The experimental design of the microarrays could not be a full loop design since the generalist *N. albigula* cannot consume a diet of 70% juniper without significant weight loss. Also for the between treatment comparison, the specialist *N. stephensi* was not directly compared on the control versus 70% juniper diet. We felt that a 25% juniper diet was more ecologically relevant and therefore more interesting than the control diet and it is recommended for array studies to make direct comparisons between samples of highest interest (Churchill, 2002). Hybridized microarrays were washed according to the manufacturer's protocol and scanned on an Agilent Technologies G2565AA Microarray Scanner System with SureScan technology. Ratio data were extracted from scanned microarray images using Feature Extraction 5.1.1 software (Agilent Technologies), and dye-normalized, background-subtracted intensity data were exported to text and GEML-format files.

2.6. Data analysis

Background-subtracted intensity data were $\log_2$ transformed and imported into SAS. A mixed model analysis of variance was used to determine which genes differed significantly in expression between the treatment groups. A normalization and gene model were used as described in Wolfinger et al. (2001). The normalization model contained dye as a fixed effect and array and array * dye as random effects. The residuals from the normalization model were used in the gene model. The gene model contained treatment, dye and treatment * dye as fixed effects and array as a random effect. The differences of the least square means between treatments and the associated $\log_{10} p$-values were used to construct volcano plots. Genes were considered significant if the least square mean difference between treatments was $p < 0.001$. The reduced significance level is applied to microarray experiments to reduce Type I errors among the vast number of comparisons. The reduced significance level could be attained with our small sample sizes because of technical replication with each sample being represented on more than one array in order to attain a semi-loop design and dye swapping.

To test the hypothesis that the specialist relies more heavily on phase I versus phase II enzymes, we used the $\log_2$ transformed background subtracted intensity data as a proxy for the expression levels of genes encoding phase I or phase II enzymes. A total of 112 phase I genes and 69 phase II genes were present as transcripts on the chip. We considered these 181 genes as probable detoxification genes in the woodrats. The cumulative expression of phase I versus phase II was determined by summing the expression levels of the genes identified as phase I versus phase II for each treatment group. The average expression levels for phase I versus phase II were determined by dividing the cumulative expression for phase I versus phase II by the number of genes in each group. We analyzed both total cumulative expression for each phase as well as average expression levels using an ANOVA with species, diet and phase as fixed effects.

3. Results

3.1. Between species differences

There were considerable differences in constitutive gene expression between the species (Fig. 1a). A total of 225 genes differed in expression ($p < 0.001$) with 114 genes showing higher expression in *N. albigula* and 111 genes showing higher expression in *N. stephensi* fed the terpene-free diet (Table 2). There were significant differences in expression levels of eleven detoxification genes (Table 3).

The pattern of gene expression differences between *N. albigula* and *N. stephensi* fed the 25% juniper diet was similar to the between species pattern on the terpene-free diet in that many genes showed large as well as significant differences (Fig. 1b). However, significantly more genes differed between woodrat species on the 25% juniper compared to the control (506 genes at $p < 0.001$). *Neotoma albigula* had higher expression than *N. stephensi* for 260 genes, while *N. stephensi* had higher expression of 246 genes when both species were fed 25% juniper diet (Table 2). A total of 22 detoxification genes had significant differences in expression levels (Table 3).

Table 2
Number of genes differentially expressed at $p < 0.001$

Between species			
	Total	↑ *N. albigula*	↑ *N. stephensi*
Terpene-free diet: *N. albigula* versus *N. stephensi*	225	114	111
25% juniper diet: *N. albigula*versus *N. stephensi*	506	260	246
Between treatments			
	Total	↑ Terpene-free	↑ 25% juniper
N. albigula: terpene-free versus 25% juniper diet	30	15	15
N. stephensi: terpene-free versus 25% juniper diet	25	15	10
	Total	↑ 25% juniper	↑ 70% juniper
N. stephensi: 25% juniper versus 70% juniper diet	740	361	379

Table 3
Genes differentially expressed that play a role in detoxification

Between species

Terpene free diet: *N. albigula* versus *N. stephensi*

Description	Fold difference[a]	Class	GenBank ID
Cytochrome P450 olfl	6.47+/−0.23	Phase I	M33296.1
Cytochrome P450 2B	4.79+/−0.46	Phase I	AF159245.1
Glutathione peroxidase	2.76+/−0.34	Phase I	M21210.1
Cytochrome P450 2D2	−2.15+/−0.24	Phase I	NM_012730
Catecholamine-*O*-methyltransferase	5.20+/−0.27	Phase II	NM_012531
Heparan sulfate 6-sulfotransferase 1	1.72+/−0.25	Phase II	H31316
Uridindiphosphoglucosepyrophosphorylase 2	−2.08+/−0.28	Phase II	AI231301
Glutatione-*S*-transferase 2	−3.03+/−0.31	Phase II	NM_017013
Nuclear receptor 1h4	2.43+/−0.23	Nuclear receptor	NM_021745
Solute carrier 22a1	2.65+/−0.38	Transporter	NM_012697
Solute carrier 35	0.82+/−0.10	Transporter	AW434979

25% juniper diet: *N. albigula* versus *N. stephensi*

Description	Fold difference[a]	Class	GenBank ID
Cytochrome P450 2B	4.60+/−0.42	Phase I	AF159245.1
Cytochrome P450 4F14	3.05+/−0.43	Phase I	NM_019623
Glutathione peroxidase	2.74+/−0.31	Phase I	M21210.1
Cytochrome P450 3A3	2.41+/−0.31	Phase I	NM_013105
Cytochrome P450 olfl	2.17+/−0.22	Phase I	M33296.1
Cytochrome P450 2F1	−0.94+/−0.14	Phase I	NM_019303
Glutathione reductase	−1.23+/−0.19	Phase I	NM_053906
Monoamine oxidase B	−1.74+/−0.14	Phase I	NM_013198
Cytochrome P450 2D2	−1.85+/−0.22	Phase I	NM_012730
Aldehyde dehydrogenase 1A4	−2.09+/−0.23	Phase I	NM_017272
Catecholamine-*O*-methyltransferase	6.26+/−0.26	Phase II	NM_012531
UDP-glucuronosyltransferase 2	2.16+/−0.31	Phase II	NM_173295
Heparan sulfate 6-sulfotransferase 1	1.89+/−0.24	Phase II	H31316
Protein-tyrosine sulfotransferase 1	−1.86+/−0.17	Phase II	BF555407
Glutathione synthetase	−3.79+/−0.59	Phase II	NM_012962
Glutathione-*S*-transferase 2	−3.92+/−0.29	Phase II	NM_017013
Nuclear receptor 1H4	3.07+/−0.22	Nuclear receptor	NM_021745
Hepatocyte nuclear factor 4α7	1.27+/−0.17	Nuclear receptor	AF329936.1
Solute carrier 22a1	3.90+/−0.36	Transporter	NM_012697
Organic cation transporter 22a1	3.48+/−0.36	Transporter	NM_012697
Solute carrier 35	1.54+/−0.20	Transporter	BF396589
Solute carrier 35	0.78+/−0.09	Transporter	AW434979

Between treatments

N. stephensi: terpene-free versus 25% juniper diet

Description	Fold difference	Class	GenBank ID
Glutathione peroxidase precursor	2.50+/−0.31	Phase I	NM_022525
Aldehyde dehydrogenase 1a4	−1.73+/−0.23	Phase I	NM_017272
Cytochrome P450 PB1	−1.79+/−0.22	Phase I	M13711.1
Cytochrome P450 2C12	−2.03+/−0.27	Phase I	NM_012709
Cytochrome P450 olfl	−4.37+/−0.22	Phase I	M33296.1
Uridindiphosphoglucosepyrophosphorylase 2	3.04+/−0.26	Phase II	AI231301

N. stephensi: 25% juniper versus 70% juniper diet

Description	Fold difference	Class	GenBank ID
Aldehyde dehydrogenase 1a4	3.30+/−0.28	Phase I	NM_017272
Cytochrome P450 2C12	3.14+/−0.33	Phase I	NM_012709
Monoamine oxidase B	2.00+/−0.17	Phase I	NM_013198
Methylmalonate semialdehyde dehydrogenase	1.95+/−0.26	Phase I	NM_031057
Cytochrome P450 2J3	1.72+/−0.20	Phase I	U39943.1
Cytocthrome P450 olfl	−1.97+/−0.26	Phase I	M33296.1
Cytochrome P450 1A2	−2.76+/−0.42	Phase I	NM_012541
Cytochrome P450 2A3A	−2.78+/−0.43	Phase I	NM_012542
Glutathione peroxidase	−3.50+/−0.39	Phase I	M21210.1
Cytochrome P450 2B	−5.72+/−0.53	Phase I	AF159245.1
Glutathione-*S*-transferase	3.34+/−0.42	Phase II	1185280

Author's personal copy

Table 3 (*continued*)

Between species

Terpene free diet: *N. albigula* versus *N. stephensi*

Description	Fold difference[a]	Class	GenBank ID
Glutathione-*S*-transferase 2	3.00+/−0.35	Phase II	NM_017013
Glucosamine-6-phosphate acetyltransferase	0.85+/−0.13	Phase II	AI407796
Cholesterol acyltransferase	0.70+/−0.09	Phase II	NM_031118
Heparan sulfate 6-sulfotransferase 1	−3.02+/−0.28	Phase II	H31316
Catecholamine-*O*-methyltransferase	−6.95+/−0.31	Phase II	NM_012531
Hepatocyte nuclear factor 4α7	−1.65+/−0.20	Nuclear receptor	AF329936.1
Nuclear receptor 1h4	−3.74+/−0.27	Nuclear receptor	NM_021745
Solute carrier 35	−1.50+/−0.24	Transporter	BF396589
Organic cation transporter 22a1	−4.45+/−0.44	Transporter	NM_012697
Solute carrier 22a1	−5.14+/−0.44	Transporter	NM_012697

[a]Fold difference is the least-squares mean difference between treatment groups as determined by mixed-model ANOVA and is displayed as mean+/−standard error. Positive numbers mean the genes were more highly expressed in the treatment group listed first in the heading and negative numbers mean the genes were more highly expressed in the treatment group listed second in the heading.

3.2. Between treatment differences

The within species differences in gene expression between animals fed the terpene-free and 25% juniper diet were minimal for both *N. albigula* and *N. stephensi* (Fig. 1c and d). However, numerous differences in gene expression occurred between *N. stephensi* on the 25% juniper and 70% juniper diet (Fig. 1e). A total of 30 genes showed expression differences in *N. albigula* fed the terpene-free versus 25% juniper diet, with 15 genes having higher expression in animals fed the terpene-free diet and 15 with higher expression in animals fed the 25% juniper diet (Table 2). No detoxification genes had significant expression differences. The total number of genes with expression differences between *N. stephensi* fed the terpene-free diet and 25% juniper diet was 25, with 15 genes having higher expression in control fed *N. stephensi* and 10 genes having higher expression in 25% juniper fed *N. stephensi* (Table 2). Six detoxification genes had significant expression differences (Table 2).

The pattern of gene expression differences between *N. stephensi* fed 25% juniper and 70% juniper was more similar to the pattern observed in the between species comparisons rather than the other between diet comparisons (Fig. 1e). Numerous genes showed large as well as significant differences. A total of 740 genes differed significantly in expression with 361 genes showing higher expression in *N. stephensi* fed 25% juniper and 379 genes showing a higher expression in *N. stephensi* fed 70% juniper (Table 2). Twenty-one of the genes expressed differently were detoxification genes (Table 3).

Neotoma stephensi on 25% and 70% juniper diets up-regulated more genes encoding for phase I enzymes than phase II enzymes compared to *N. stephensi* on the terpene-free diet (Table 4). In contrast, *N. albigula* fed the 25% juniper diet did not up-regulate any genes encoding for phase I or phase II enzymes compared to *N. albigula* fed the terpene-free diet (Table 4).

3.3. Expression of Phase I versus Phase II genes

The woodrats in all treatment groups had higher cumulative as well as average expression of phase I versus phase II genes (Fig. 2a, b). The cumulative expression of phase I genes was not affected by species ($F_{1,15}=0.537$, $p=0.475$). However, there was a significant effect of diet ($F_{2,15}=4.789$, $p=0.025$). *Neotoma stephensi* fed the terpene-free diet had higher cumulative expression of both phase I as well as phase II enzymes compared to *N. stephensi* fed 25% and 70% juniper diets (Fig. 2a). There were no significant differences between *N. albigula* fed terpene-free and 25% juniper diet in the cumulative expression of both phase I and phase II enzymes (Fig. 2a). Like the cumulative expression, the average expression of phase I versus phase II enzymes was not affected by species ($F_{1,3675}=2.634$, $p=0.105$) but was affected by diet ($F_{2,3675}=17.650$ $p<0.001$). Both *N. stephensi* and *N. albigula* fed the terpene-free diet had higher average expression of both phase I as well as phase II enzymes than those fed 25% juniper (Fig. 2b). *Neotoma stephensi* fed the 70% juniper diet had the lowest average expression of both phase I as well as phase II enzymes (Fig. 2b).

4. Discussion

To consume large quantities of a single species of plant, mammalian herbivores process large doses of potentially toxic PSCs. Juniper contains high levels of terpenes that are readily absorbed and quite toxic. Yet one rodent species, *N. stephensi* specializes on juniper, while a sympatric species, *N. albigula* consumes far less juniper. To investigate the mechanisms of detoxification in these two species, we used microarrays to examine the hepatic gene expression of *N. albigula* and

Table 4

Number of phase I and phase II genes up regulated

	Between treatments		
	N. albigula Terpene-free versus 25% juniper	*N. stephensi* Terpene-free versus 25% juniper	*N. stephensi* 25% juniper versus 70% juniper
Phase I	0	4	5
Phase II	0	0	2

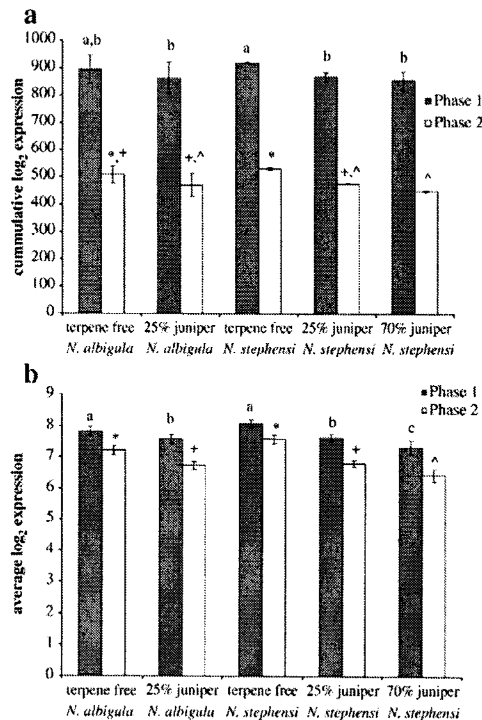

Fig. 2. a–b: Cumulative (a) and average (b) expression of genes encoding for phase I versus phase II detoxification enzymes. Phase I cumulative as well as average expression is higher than phase II in all treatment groups (cumulative: $F_{1,15}=557.517$ $p<0.001$, average: $F_{1,3675}=60.174$ $p<0.001$). Letters a, b and c denote means that are significantly different ($p<0.05$) within phase I and symbols *, + and ^ denote means that are significantly different ($p<0.05$) within phase II as determined by Tukey's HSD.

N. stephensi fed terpene-free and juniper diets. We found that the two species regulate hepatic gene expression differently, both constitutively as well as when exposed to juniper. We also tested the hypothesis that the specialist, *N. stephensi*, relies on less costly phase I enzymes more than phase II detoxification enzymes compared to the generalist. Although we found that *N. stephensi* had higher cumulative as well as average expression of phase I versus phase II enzymes, the generalist *N. albigula* had a similar pattern of enzyme expression. In the following paragraphs, we first discuss the use of microarrays for this experiment. We then discuss the pattern of gene expression in the livers of a specialist versus generalist species of woodrat. We discuss in more detail the specific detoxification genes that were up and down regulated in *N. stephensi* fed 70% juniper diet that may be integral to detoxifying PSCs found in juniper. Finally, we discuss our results with regard to the hypothesis that specialists use phase I enzymes more extensively than phase II.

4.1. The use of microarrays

Microarrays are an extremely powerful genomic technique that allow for the simultaneous analysis of the expression of thousands of genes. By analyzing thousands of genes at once it is possible to get a snapshot of whole cell or whole organ gene regulation. While microarrays have been widely used in biomedical fields, they are rarely utilized in ecological studies since most commercially available arrays are made for model organisms (Gibson, 2002; Gracey and Cossins, 2003; Held et al., 2004; Thomas and Klaper, 2004; Lettieri, 2006). Because developing a microarray for a non-model species can be a resource and time-consuming endeavor, researchers have instead successfully used microarrays developed for a model species on their species of interest (Enard et al., 2002; Miller et al., 2002; Shah et al., 2004; Brodsky et al., 2005).

For this study, we chose to use a commercially available microarray for *Rattus norvegicus* because *Neotoma* is closely related to *Rattus* in that they are in the same family, Muridae. Cross-species hybridizations have been used in a number of microarray studies (Enard et al., 2002; Miller et al., 2002; Shah et al., 2004; Brodsky et al., 2005). Enard et al. (2002) compared two groups of closely related species (humans, chimpanzees, orangutans, rhesus macaques and *Mus spretus*, *M. caroli*, *M. musculus*) using cross-species hybridizations to determine how viable the technique is to study the role of gene expression changes in brain evolution. They found that not only do closely related species have numerous differences in gene expression, similar to our study, but that cross-species hybridization is an accurate method for studying species specific patterns of gene expression.

4.2. Differences in gene expression patterns

The specialist, *N. stephensi*, and generalist, *N. albigula*, regulate hepatic gene expression differently both constitutively as well as in response to juniper consumption (Fig. 2a–e). These patterns of gene expression were mirrored in the number of genes that were expressed differently from each other at the $p<0.001$ level (Table 1). The between species comparisons of woodrats on the terpene-free diet (Fig. 2a) and the 25% juniper diet (Fig. 2b) show a pattern characteristic of volcano plots with many genes showing high fold difference in expression as well as high statistical significance. A similar pattern of differences in gene expression occurred between *N. stephensi* fed 25% juniper diet and 70% juniper diet (Fig. 2e). In contrast, a different pattern emerged within species fed terpene-free and 25% juniper diets. Both the specialist and generalist displayed very little differences in gene expression on a terpene-free or 25% juniper diet as evidenced by the oval shaped volcano plots (Fig. 2c, d).

In the case of the specialist, *N. stephensi*, it is possible that a diet of 25% juniper is too low to induce or suppress detoxification genes. *Neotoma stephensi* absorbs less alpha-pinene than the generalist, *N. albigula* (Sorensen et al., 2004); thus the absorbed dose of terpenes from the 25% juniper diet may in fact be too low to cause changes in gene regulations in *N. stephensi*. For *N. albigula*, the generalist, a 25% juniper diet is comparable to their ecologically relevant diet of juniper (Dial, 1988). The relatively few changes in gene expression that occur may indicate that the generalist is not able to further induce detoxification with respect to juniper consumption. Therefore,

the generalist may only be able to consume low levels of juniper (25% of its diet) because that is the largest dose of juniper it can effectively detoxify with its constitutive level of hepatic enzymes. The specialist, *N. stephensi*, in contrast had a large change in gene regulation when exposed to its ecologically relevant dose of juniper (70%, Dial, 1988). Therefore, it seems that *N. stephensi*'s ability to specialize on juniper is not due to constitutive levels of gene expression but rather that it can respond to high levels of juniper with large changes in hepatic gene regulation.

4.3. Detoxification genes involved in juniper detoxification

A number of detoxification genes showed significant differences in expression level both between as well as within species (Table 2). There were few genes, detoxification or otherwise that were significantly different within species for both the specialist and generalist when on a control or 25% juniper diet. In contrast, far more genes, were differentially regulated between the specialist fed a 25% juniper versus 70% juniper diet. The phase I genes that were up-regulated in the *N. stephensi* fed the 70% juniper diet were five cytochrome P450s (CYP), CYP olfl, CYP 1A2, CYP 2A, CYP 3A, and CYP 2B as well as glutathione peroxidase. Cytochrome P450s are heme-containing proteins that introduce hydroxyl groups into the structures of their substrates (Danielson, 2002) and are the largest group of detoxification enzymes.

While many CYPs metabolize endogenous compounds, the five CYPs up-regulated in the specialist fed the 70% juniper diet all have exogenous compounds as substrates. Cytochrome P450 1A2 has a number of substrates, including the PSCs caffeine and warfarin (Danielson, 2002). Vegetables from the Brassicaeae family such as broccoli and Brussels sprouts are known inducers of CYP 1A2 (Butterweck et al., 2004). Thus, it is not unreasonable to assume that some of the terpenes or phenolics present in juniper are also inducers of CYP 1A2. The CYP 2A family in humans also has PSCs as substrates, e.g., nicotine and coumarin (Xu et al., 2002). Similarly to CYP 1A2, it is likely that some of the terpenes or phenolics present in juniper are inducers of CYP 2A. Cytochrome P450 2B was the most highly up-regulated CYP. It was 5.7× higher in *N. stephensi* fed 70% juniper compared to *N. stephensi* fed 25% juniper. Phenobarbital induces CYP 2B in humans and rats (Danielson, 2002) and based on our results, high levels of juniper cause induction of CYP 2B in *N. stephensi*.

Interestingly CYP olfl, a putative olfactory specific gene in laboratory rats was up-regulated almost 2× in *N. stephensi* fed 70% juniper. Cytochrome P450 olfl is an olfactory specific CYP in laboratory rats with a proposed function of detoxifying inhaled chemicals (Nef et al., 1989). Terpenes, the major class of PSCs found in juniper are highly volatile and therefore easily inhaled. Currently, there are no known substrates for CYP olfl, but its up-regulation in *N. stephensi* on a 70% juniper diet suggests terpenes may be substrates for CYP olfl. Unlike laboratory rats, CYP olfl is not olfactory specific in woodrats, perhaps because they regularly consume volatile PSCs. It would be interesting to determine if CYP olfl would be expressed in the livers of laboratory rats fed volatile compounds such as terpenes.

Glutathione peroxidase was another phase I gene up-regulated >3× in *N. stephensi*. Glutathione peroxidase is a peroxide scavenging enzyme that plays an important role in the cellular defense mechanisms against oxidative stress (Miyamoto et al., 2003). When juniper is consumed in large quantities, either the PSCs directly or the metabolites, may create reactive oxygen species necessitating up regulation of enzymes such as glutathione peroxidase to guard against oxidative stress.

A number of phase I genes were down-regulated in the *N. stephensi* fed 70% juniper compared to 25% juniper. Aldehyde dehydrogenase 1a4 (ALDH1a4) CYP 2C12, CYP 2J3 as well as monoamine oxidase B (MOAB) and methylmalonate semialdehyde dehydrogenase (MMSDH) were down regulated. It is possible that the PSCs present in juniper inhibit these enzymes or their metabolites have negative feedback on the production of these enzymes.

Two phase II genes were up-regulated in *N. stephensi* fed 70% juniper. Heparan sulfate 6-sulfotransferase 1 (HS6ST1) was up-regulated >3× and catecholamine-*O*-methyltransferase (COMT) was up-regulated almost >7×. Heparan sulfate-6-sulfotransferase is involved in the metabolism of heparan sulfate that plays an integral role in blood coagulation (Habuchi et al., 1998). While the up-regulation of HS6ST1 may not play a direct role in detoxifying the PSCs in juniper, the conjugation of a sulfate group to form heparin sulfate does not represent an energetic loss, since the heparan sulfate is not excreted. Anecdotally, the blood of the specialist, *N. stephensi*, seems to coagulate more quickly than other woodrat species (Skopec personal observation) and perhaps there is a link between juniper intake and coagulation mitigated through the up-regulation of HS6ST. The other phase II enzyme up-regulated was COMT, which conjugates a methyl group to its substrate. The loss of a methyl group as a conjugate may be less energetically costly than glucose or amino acid conjugates used by other phase II enzymes.

Two glutathione-*S*-transferase (GST) genes were down-regulated more than 3× in *N. stephensi* fed 70% juniper. The loss of a glutathione conjugate, a tripeptide, would be potentially costly to a wild herbivore consuming a low nitrogen diet. Juniper is low in nitrogen, yet *N. stephensi* is able to maintain nitrogen balance and even increase in mass on a high juniper diet (Dearing et al., 2005). Therefore, down-regulating phase II enzymes that result in the loss of nitrogen, such as GST, may allow *N. stephensi* to specialize on a plant low in nitrogen, and high in PSCs like juniper. Further study of the regulation and substrate usage of the detoxification enzymes up and down-regulated by *N. stephensi* in response to the 70% juniper diet will reveal their importance in the detoxification of PSCs present in juniper.

4.4. Phase I versus II

We tested the hypothesis that specialists rely on phase I to a greater extent than on phase II by analyzing the total expression of all the genes encoding phase I or phase II detoxification

enzymes on the microarray. The cumulative and average expression of phase I versus phase II enzyme genes (Fig. 2a, b) showed less variability than the results of comparing individual genes (Table 3) because increased expression of a particular detoxification gene in a specific treatment group (e.g., COMT in 70% juniper, *stephensi*) was balanced out by an equal or larger decrease in expression of other detoxification gene or genes in the same treatment group.

The cumulative expression of phase I enzymes was greater than phase II enzymes in both species in all treatment groups (Fig. 2a). However, an internal bias exists in the array in that there were more phase I transcripts (112) than phase II enzyme transcripts (69) due to the fact that there are more known phase I genes than phase II genes (Klaassen, 2001). We, therefore, also analyzed the average expression of phase I versus phase II genes to remove the effect of the different number of genes for each group present on the microarray. The average expression of phase I versus phase II genes showed a similar pattern as the cumulative expression, with phase I average expression being higher in both species in all treatment groups (Fig. 2b). While there was no difference between the species on the same diet, there was a significant effect of diet on the cumulative as well as average expression of phase I versus phase II enzymes. The specialist, *N. stephensi* had lower cumulative expression of phase I as well as phase II on the juniper diets (25% and 70%) compared to *N. stephensi* on the terpene-free diet, while there was no significant difference between *N. albigula* fed either the 25% juniper diet or the terpene-free diet (Fig. 2a). Thus, it seems that the specialist, *N. stephensi*, may down-regulate its detoxification system as a whole in response to the presence of juniper in its diet. Similarly, to the cumulative of phase I and phase II genes *N. stephensi* also decreased the average expression of both phase I as well as phase II genes in response to the 25% as well as 70% juniper diets.

It is commonly thought that exposure to toxic PSCs, as one might expect a juniper diet to be, would cause marked up-regulation of the detoxification system. However, we found that the opposite occurred in the specialist, *N. stephensi*, on the juniper diets. The fact that *N. stephensi* consumes up to 70% of its diet as juniper both in the field and lab without any weight loss or other signs of toxicity means that the PSCs in juniper are not toxic to *N. stephensi*. Therefore, *N. stephensi* has adapted to a high juniper diet and the down-regulation of their detoxification system may be the adaptation. Down-regulation of the detoxification system may decrease energy expended for producing detoxification enzymes that do not play a role in metabolizing the PSCs present in juniper. Also down-regulating detoxification enzymes that may produce toxic metabolites of juniper PSCs may help decrease the toxicity of the juniper diets.

Neotoma albigula also significantly decreased the average expression of phase I as well as phase II genes when on the 25% juniper diet. Because no detoxification genes were expressed differently between *N. albigula* fed terpene-free and 25% juniper diet, the decrease in average expression is likely the result of slight decreases in expression of many genes. Since *N. albigula* did not have higher constitutive expression of phase II enzymes than *N. stephensi*, nor did they up-regulate the cumulative or average expression of phase II enzymes, our study does not support the hypothesis that generalists rely more heavily on phase II detoxification than specialists. It seems that both the specialist and generalist rely more heavily on phase I than phase II detoxification, and that phase I detoxification is not a novel adaptation of the specialist. However, *N. stephensi* when fed its ecologically relevant diet, down-regulated the expression of two GSTs, thereby potentially limiting the loss of a nitrogen containing conjugate. Furthermore, the specialist increased the expression of COMT, which uses a less costly methyl group as a conjugate. Overall, our results do not support the hypothesis that specialists only, rely more heavily on phase I enzymes. However, the specialist was unique in that it down-regulated some phase II enzymes that use energetically expensive conjugates in favor of other phase II enzymes that use relatively inexpensive conjugates.

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