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Liver biotransforming enzymes in woodrats *Neotoma stephensi* (Muridae)

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Abstract

Mammalian herbivores are exposed to extremely high levels of plant secondary compounds naturally present in their diet. It has been speculated that specialist herbivores should express a unique pattern of biotransforming enzymes to permit the consumption of a single species of toxic plant. Specifically, specialists should rely on pathways that effectively biotransform the toxins they routinely encounter in their diet. We examined the hepatic mRNA expression and activity or content of biotransforming enzymes in the specialist herbivorous woodrat, *Neotoma stephensi*, and compared results to those of laboratory rats (Sprague–Dawley strain *Rattus norvegicus*). In addition, we investigated the role of α-pinene, a specific plant toxin present in the diet of *N. stephensi* on the mRNA expression pattern and activity or content of biotransforming enzymes in Sprague–Dawley rats. Overall, the levels of functionalization enzyme activity and mRNA were found to be higher in specialists, while glucuronidation enzyme activity and mRNA were lower. These results support predictions that specialist herbivores rely more on functionalization biotransformation pathways rather than glucuronidation pathways.

Keywords: Biotransformation; Detoxification; Woodrats; Messenger RNA (mRNA); Cytochrome P450; Glucuronidation; UDP-glucuronosyltransferase; Alpha-pinene

1. Introduction

Mammalian herbivores are exposed to plant secondary compounds that are an inescapable part of their diet. These compounds are non-nutritive and potentially toxic molecules that herbivores must process and eliminate from the body. The metabolism of plant toxins is hypothesized to be particularly challenging for herbivores that browse on a single species of plant. Dietary specialization could result in the ingestion of large quantities of similar plant secondary compounds, overwhelming the detoxification system. Freeland and Janzen (1974) theorized that dietary specialization is constrained by the inability of species to detoxify the large quantities of similar plant toxins present in a diet comprised of a single species of plant. It is predicted that the few specialists that exist rely on an increased level of expression of specific biotransforming enzymes that allow them to effectively detoxify and eliminate high levels of plant toxins encountered.

In general, detoxification and elimination of plant toxins is carried out by a multitude of enzymes collectively referred to as "xenobiotic biotransforming enzymes" (Parkinson, 1996). These enzymes transform lipophilic molecules into hydrophilic molecules that can be excreted in the urine or feces. These enzymes are believed to have evolved in part to protect organisms from secondary compounds produced by plants (Gonzalez, 1989). Biotransforming enzymes are classified as functionalization (Phase I) or conjugation (Phase II) enzymes (Williams, 1971). Functionalization reactions include oxidation, reduction, and hydrolysis. Cytochrome P450s are a multi-gene family of mixed function oxidizes whose members make up the majority of the enzymes involved in functionalization reactions. Additional functionalization enzymes include microsomal epoxide hydrolase (mEH), and NAD(P)H quinone oxidoreductase (QOR), also known as DT-diaphorase (Parkinson, 1996).

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Functionalization reactions can lead to direct inactivation of a hydrophobic compound, or they can create a reactive metabolite, requiring conjugation reactions for complete elimination.

Conjugation incurs an energetic cost, requiring that high-energy cofactors be replenished through dietary sources (Parkinson, 1996). During conjugation, hydrophilic acceptor groups are transferred to the functional groups of available compounds. These reactions synthesize a more polar compound that is easily removed from the cell. Conjugation enzymes include a family of enzymes referred to as UDP-glucuronosyltransferases (UGTs). UGT's conjugate chemical compounds with the sugar acid moiety from UDP-glucuronic acid (UDPGA). Glucuronidation results in the excretion of glucuronidated compound at the cost of a glucose molecule.

It is theorized that the levels of expression of specific biotransforming enzymes may facilitate dietary specialization in mammals. Specifically, specialists may possess high levels of biotransformation enzymes that enhance excretion of the particular secondary compounds present in a single plant species, thereby increasing tolerance to that plant. Additionally, specialists may rely on biotransformation pathways that are less energetically expensive, thereby minimizing the costs of detoxification. For example, the koala (*Phascolarctos cinereus*), a specialist herbivore, generates more oxidized metabolites than conjugation metabolites. In addition, koalas generate more oxidized metabolites than generalist marsupial species exposed to the same plant secondary compound (Boyle et al., 2001; Pass et al., 2001; Stupans et al., 2001). Furthermore, previous studies by our group demonstrated a higher expression of hepatic functionalization enzyme mRNAs over glucuronidation enzyme mRNAs in a population of *N. lepida* woodrats that consumes large amounts of the toxic plant, *Juniperus osteosperma* (Lamb et al., 2001). Although these data suggest that reliance on functionalization enzymes facilitates consumption of toxic plants, the expression of various biotransformation enzyme pathways has not been investigated in specialist mammalian herbivores.

We investigated the biotransforming capacity of a specialist herbivore, *Neotoma stephensi*, that specializes on one seeded juniper (*Juniperus monosperma*). An extensive multi-year study revealed that the diet of *N. stephensi* was composed of up to 96% juniper (Dial, 1988). Juniper contains high quantities of phenolics and terpenes, specifically monoterpenes. α-Pinene comprises up to 55–65% of the monoterpenes in *J. monosperma* (Dearing et al., 2000). Furthermore, α-pinene exerts numerous deleterious effects on humans and laboratory rats including CNS depression, mucous membrane irritation and nephritis and renal failure (Koppel et al., 1981). Each day specialist *N. stephensi* consumes the equivalent to half the lethal human-scaled dose of terpenes (Koppel et al., 1981). *N. stephensi* is more tolerant to one-seeded juniper and α-pinene than a related generalist woodrat (*N. albigula*) and therefore willingly consumes more juniper and α-pinene than generalists (Dearing et al., 2000). Furthermore, *N. stephensi* maintained acid–base homeostasis, an indicator of detoxification (Foley et al., 1995), better than generalists when exposed to similar loads of α-pinene (Dearing et al., 2000). The fact that *N. stephensi* woodrats have a higher tolerance and lower toxic response to juniper and α-pinene indicates that they may be more efficient at metabolizing juniper toxins than generalist woodrats.

We tested the emerging theory of herbivore specialization that specialist species have evolved an increased capacity for P450 oxidation and a decreased capacity for conjugation with glucuronides (Boyle et al., 2001; McLean et al., 1993) using *N. stephensi* woodrats. We examined hepatic expression of enzyme activities and mRNAs for several biotransforming enzymes in wild *N. stephensi* woodrats and compared them to those in laboratory rats (Sprague–Dawley). To investigate whether *N. stephensi* has a reduced capacity of glucuronidation conjugation, we examined the hepatic UGT activity of *N. stephensi* towards three model substrates. We also compared the regulation of hepatic biotransforming enzymes and mRNAs in laboratory rats treated with α-pinene to those found in wild *N. stephensi* woodrats. This comparison was an initial attempt to determine the role of a single toxic component in the diet of *N. stephensi* on the expression of specific biotransforming enzymes and mRNAs.

2. Materials and methods

2.1. Chemicals

p-Nitrophenol, 1-naphthol, pentoxyresorufin, α-pinene and UDP-glucuronic acid (UDPGA) and other biochemicals were purchased from Sigma (St. Louis, MO, USA). TRIzol solution for total RNA isolation was purchased from Invitrogen (Carlsbad, CA, USA). Nytran membranes were purchased from ISC Bioexpress (Kaysville, UT, USA), and the Multiprime DNA labeling kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). [α-^{32}P]dCTP was purchased from DuPont NEN (Boston, MA, USA).

2.2. Laboratory rats

Male laboratory rats (Muridae: Murinae: *Rattus norvegicus*: strain "Sprague–Dawley"; 160–180 g) were obtained from Simonson laboratories and kept on a 12-h light/dark cycle and given free access to rat chow and water. Four Sprague–Dawley rats were not given any chemical treatment and are referred to as "control rats". Four Sprague–Dawley rats were gavaged with 200 μl α-pinene in 200 μl corn oil (~1000 mg/kg) orally, once daily for 3 days, and sacrificed 24 h after the last dose. Sprague–Dawley rats treated with α-pinene are referred to as "α-pinene treated

rats". The α-pinene dose given to the α-pinene treated rats approximates the amount of α-pinene ingested daily by wild *N. stephensi* woodrats (Dearing et al., 2000). No behavioral toxicity was observed.

2.3. Collection of neotoma

N. stephensi (Muridae: Sigmodontinae) were collected in October of 1999 (IACUC no. 98-02011). Four female woodrats were collected from a site in Woodhouse Mesa, AZ [35°30′ N 111°27′ W]. For a description of this site see Dearing et al. (1998). Animals were live-trapped with Sherman traps, and whole animal body mass was recorded. Trapped animals were placed in shoebox cages (48×27×20 cm) with unlimited access to fresh *J. monosperma* and a small amount of rabbit chow (Harland Teklad formula 2120). *J. monosperma* was collected from trees surrounding the trapping site and kept on dry ice to maintain chemical composition of plant material. Woodrats were transported to the University of Utah animal facility and were sacrificed within 3 days of capture. During these 3 days, we verified that woodrats were consuming fresh juniper and therefore, assume that they continued to maintain enzyme capacity prior to liver collections.

2.4. Microsomal preparation and enzyme assays

Microsomal and cytosolic fractions from saline infused livers were prepared according to the procedure described by Franklin and Estabrook (1971). Protein concentrations were determined by the method of Lowry et al. (1951). Overall cytochrome P450 concentrations were determined from microsomes using the carbon monoxide difference spectrum by the method of Omura and Sato (1964). Pentoxyresorufin *O*-deethylase (PROD) activity was determined by the method of Lubet et al. (1985). QOR activities were determined by the dicoumarol-inhibited reduction 2,6-dichlorophenolindophenol by NADH at pH 7.4 as described by Benson et al. (1980). UGT activities for 4-nitrophenol were determined as described by Ritter and Franklin (1987), 1-naphthol and morphine activities were determined as described by Liu and Franklin (1984).

2.5. Total RNA isolation and northern blot analysis

Total RNA was isolated from approximately 100 mg of frozen liver tissue by homogenization in 2 ml TRIzol (phenol-guanidine isothiocyanate) reagent (Gibco/BRL) and isopropyl alcohol precipitation of the aqueous layer following chloroform treatment. Twenty micrograms of total RNA from each sample (concentration of total RNA was determined from 260 nm absorbance) was subjected to electrophoresis in 1% denaturing agarose gel containing formaldehyde. Sample integrity was determined by visualization of sharp 18S and 28S bands with ethidium bromide. Total RNA was transferred to a Nytran membrane (Schleicher and Schuell) by downward alkaline transfer, cross-linked with UV light and hybridized with cDNA probes labeled with [α-^{32}P] dCTP by random primed synthesis (Amersham Multiprime) using the manufactures' specifications. Rat cDNA probes for the various biotransformation enzyme mRNAs were used as described previously (Lamb et al., 2001).

Hybridized blots were washed twice for 30 min at 42 °C in 2× SSC, 0.1% SDS, twice for 30 min at 42 °C in 0.1× SSC, 0.1% SDS, and once for 45 min at 54 °C in 0.1× SSC, 0.1% SDS. Autoradiographic film was exposed for 6–72 h at −70 °C with an intensifying screen and the developed band intensity was determined by scanning densitometry using Molecular Analyst (Bio-Rad) software. To remove any effects of gel loading, and transfer variations, all mRNA bands were normalized to a housekeeping gene, cyclophilin mRNA, in the same sample.

2.6. Statistics

Expression level of each biotransformation enzyme and mRNA was compared between each group: wild *N. stephensi* (NS), control rats, and α-pinene treated rats using ANOVA, with groups as the main effect. ANOVAs were followed by Fisher's multiple range test. Significance was determined using a p-value less than 0.05.

3. Results

3.1. Functionalization capacity

3.1.1. Hepatic P450 levels, PROD and QOR enzyme activities

The level of P450, PROD and QOR enzyme activities in wild *N. stephensi* liver were compared to that of control and α-pinene treated Sprague–Dawley rat liver (Table 1). The overall level of cytochrome P450 in *N. stephensi* liver was approximately two fold higher than control rat and α-pinene treated rat liver. There was no change in overall P450 levels

Table 1
Level of P450, PROD and QOR enzyme activities in wild *N. stephensi* liver compared to that of control and α-pinene treated Sprague–Dawley rat liver

Enzyme	Group		
	N. stephensi	α-Pinene SD	Control SD
Overall			
P450 (nmol/mg)	1.74±0.25[a]	0.74±0.12[b]	0.78±0.05[b]
PROD (nmole/min/mg)	0.16±0.02[a]	0.28±0.01[b]	0.05±0.02[c]
QOR (nmol/mg/min)	161±42.5[a]	383±29.4[b]	147±7.40[a]

Hepatic microsomal enzyme activity of cytochrome P450, PROD, and cytosolic QOR in *Neotoma stephensi* (*N. stephensi*, n=4), Sprague–Dawley rats treated with α-pinene (α-pinene SD, n=4) and untreated Sprague–Dawley rats (control SD, n=4). Parameters determined are given as the mean±SE. Different letters indicate significant differences between groups at the p<0.05 level.

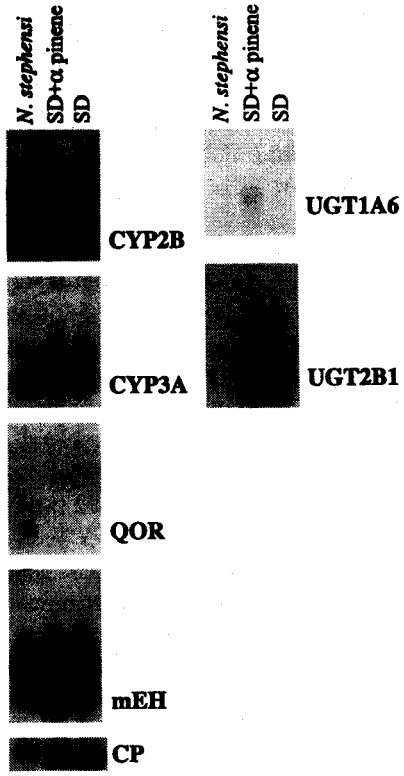

Fig. 1. Representation of Northern blot analysis of hepatic mRNA in *Neotoma stephensi* compared to that of control and α-pinene treated rats. SD+pinene (Sprague–Dawley rats treated with α-pinene); SD (untreated Sprague–Dawley rats); CP (housekeeping gene, cyclophilin).

in α-pinene treated rats over control rats. The level of PROD activity was 3.2-fold higher in *N. stephensi* liver than control rat liver. PROD activity in α-pinene treated rat liver was 43% higher than wild *N. stephensi* liver and 5.6-fold higher than control rats. Levels of QOR enzyme activities in α-pinene treated rat liver were higher than both *N. stephensi* and control rats.

Table 2
Level of expression of CYP2B, CYP3A, QOR and mEH mRNAs in *N. stephensi* woodrats compared to that of control and α-pinene treated rats

mRNA	Group		
	N. stephensi	α-Pinene SD	Control SD
CYP2B	18.3±5.38[a]	1.98±0.75[b]	1.00±0.39[b]
CYP3A	8.24±2.91[a]	1.29±0.16[b]	1.00±0.09[b]
QOR	19.8±6.52[a]	0.90±0.19[b]	0.97±0.31[b]
mEH	3.31±0.47[a]	1.34±0.20[b]	1.00±0.15[b]

Alterations of hepatic functionalization mRNAs in *Neotoma stephensi* (*N. stephensi*, n=4), Sprague–Dawley rats treated with α-pinene (α-pinene SD, n=4) and untreated Sprague–Dawley rats (control SD, n=4). The mean Northern Blot density for untreated Sprague–Dawley animals was arbitrarily set as 1, and samples from treated Sprague–Dawley rats and *N. stephensi* woodrats are expressed as increases or decrease for that value. Parameters determined are given as the mean±SE. Different letters indicate significant differences between groups (p<0.05).

Table 3
Levels of hepatic microsomal UGT activities in *N. stephensi* compared to that of control and α-pinene treated rats

UGT substrate	Group		
	N. stephensi	α-Pinene SD	Control SD
p-Nitrophenol	6.77±1.18[a]	11.4±0.22[b]	8.49±1.50[a]
1-Naphthol	23.5±8.67[a]	49.8±6.78[b]	48.8±1.25[b]
Morphine	3.70±1.19[a]	18.4±4.56[b]	6.95±0.49[a]

Hepatic microsomal UGT activities in *Neotoma stephensi* (*N. stephensi*, n=4), Sprague–Dawley rats treated with α-pinene (α-pinene SD, n=4) and untreated Sprague–Dawley rats (control SD, n=4). Parameters determined are given as the mean±SE. Different letters indicate significant differences between groups (p<0.05). Activities are reported as nmol/mg/min.

3.1.2. Northern blot analysis of hepatic CYP1A1, CYP2B, CYP3A, QOR and mEH mRNAs

CYP1A1 mRNA was undetectable in *N. stephensi* woodrat liver (data not shown). The level of expression of CYP2B, CYP3A, QOR and mEH mRNAs in *N. stephensi* woodrats were several times higher than control and α-pinene treated rats (Fig. 1 and Table 2). Treatment of Sprague–Dawley rats with α-pinene did not cause a significant change in expression in CYP2B, CYP3A, QOR or mEH mRNAs from control rats.

3.2. Conjugation enzymes

3.2.1. Hepatic UGT enzyme activities

The level of hepatic UGT activity in *N. stephensi* liver were compared to control and α-pinene treated Sprague–Dawley rat liver (Table 3). UGT activity for *p*-nitrophenol of woodrats and control rats did not differ, but activity in *N. stephensi* and control rats was lower than in α-pinene treated rats. The level of hepatic 1-naphthol glucuronidation in *N. stephensi* woodrats was approximately 50% of the level in control and α-pinene treated rats. Morphine glucuronidation in *N. stephensi* woodrats was fivefold lower than α-pinene treated rats, but did not differ significantly from control rats. Sprague–Dawley rats treated with α-pinene had higher UGT activity towards *p*-nitrophenol and morphine over control rats. There was no effect

Table 4
Level of expression of hepatic UGT1A6 and UGT2B1 mRNAs in *N. stephensi* compared to that of control and α-pinene treated rats

mRNA	Group		
	N. stephensi	α-Pinene SD	Control SD
UGT1A6	2.71±0.78[a]	2.07±0.61[a]	1.00±0.20[b]
UGT2B1	n.d.	2.25±0.24[a]	1.00±0.17[b]

Alterations of conjugation mRNAs in *Neotoma stephensi* (*N. stephensi*, n=4), Sprague–Dawley rats treated with α-pinene (α-pinene SD, n=4) and untreated Sprague–Dawley rats (control SD, n=4). The mean Northern Blot density for untreated Sprague–Dawley animals was arbitrarily set as 1, and samples from treated Sprague–Dawley rats and *N. stephensi* woodrats are expressed as increases or decrease for that value. Parameters determined are given as the mean±SE. Different letters indicate significant differences between groups (p<0.05). n.d. indicates no detection.

of α-pinene treatment on hepatic 1-naphthol enzyme activities in laboratory rats.

3.2.2. Northern blot analysis of hepatic, UGT1A6 and UGT2B1 mRNAs

UGT1A6 mRNA in *N. stephensi* did not differ from control and α-pinene treated rats. UGT2B1 mRNA was undetectable in *N. stephensi* liver (Fig. 1 and Table 4). Treatment of Sprague–Dawley rats with α-pinene resulted in a 2.2-fold higher level of UGT2B1 mRNAs in the liver.

4. Discussion

In their seminal article on plant secondary compounds and mammalian detoxification, Freeland and Janzen (1974) proposed that 'If mammals are to eat plants containing secondary compounds, and avoid serious loss of fitness, they must possess a mechanism or mechanisms that protect them from severe physiological disturbances'. Our research focused on detoxification mechanisms that may facilitate consumption of the toxic plant, *J. monosperma* by the specialist herbivore species, *N. stephensi*. To the best of our knowledge, this is the first report of hepatic biotransformation enzyme activity and mRNA expression in *N. stephensi* woodrats and the most extensive for any specialist mammalian herbivore.

It has been hypothesized that specialist herbivores have enhanced expression of cytochrome P450s and decreased capacity for conjugation of xenobiotics with glucuronides (Boyle et al., 2000, 2001; McLean et al., 1993). For example, levels of tolbutamide hydroxylase activity, a substrate for CYP2C8/9 in humans (Relling et al., 1990), are extremely high in the eucalyptus specialists, the koala (*P. cinereus*), in comparison to Sprague–Dawley rats (Liapis et al., 2000). Our results provide further support for this hypothesis. We found that the overall level of expression of hepatic cytochrome P450 was significantly higher in the liver of *N. stephensi*, a juniper specialist, than in control Sprague–Dawley rats. Furthermore, activity of hepatic microsomal UGT was generally lower in *N. stephensi* than laboratory rats.

The increased level of hepatic functionalization enzyme activity may be the result of dietary exposure of *N. stephensi* woodrats to terpenes present in the juniper diet. P450 activity in wild woodrats are similar to those seen in previous studies in which laboratory rats were treated with essential wood oils, including α-pinene (Austin et al., 1988; Hiroi et al., 1995). In addition, induction of hepatic P450s by terpenes has been demonstrated in marsupial species (Pass et al., 1999). Although direct inhibition of P450 activity by terpenes and other chemicals have been demonstrated in vitro by other groups (Pass and McLean, 2002), it is unlikely that terpenes from the diet would remain in the microsomal preparation.

Comparisons between *N. stephensi* versus control and α-pinene treated Sprague–Dawley rats suggest that elevation of functionalization enzymes may be in part attributed to exposure to α-pinene. For example, PROD enzyme activity was significantly higher in *N. stephensi* and α-pinene treated Sprague–Dawley rats than control rats. QOR, another functionalization enzyme, was approximately three-fold higher in α-pinene treated rats than control rats. In agreement, QOR mRNA was nearly 19-fold higher in *N. stephensi* than in Sprague–Dawley rats. Although similarities in functionalization capacity exist between *N. stephensi* and α-pinene treated Sprague–Dawley rats, there are also several discrepancies within specific enzymes and between enzyme activity and mRNA.

Discrepancies between functionalization activity in *N. stephensi* and α-pinene treated Sprague–Dawley rats may be an affect of diet. *N. stephensi* consuming juniper ingests a diverse mixture of plant toxins that may induce biotransformation activity differently than a single toxin. For example, two populations of *N. lepida* foraging on different plant species exhibit different biotransformation patterns (Lamb et al., 2001). Furthermore, there may be inherent differences in biotransformation patterns between species as was demonstrated between *N. lepida* and Sprague–Dawley rats (Lamb et al., 2001). We are currently investigating hepatic expression of enzyme activities and mRNAs for several biotransforming enzymes in Sprague–Dawley rats and other woodrat species given single toxins and whole plant foliage to better understand the effects of diet and species.

Previous studies on dietary specialization in several species of marsupial herbivores have lead to the proposal that specialists have a decreased capacity for glucuronidation enzyme activity. In general, our results support this hypothesis and are in agreement with other studies. For example, our group has previously found that the expression of various conjugation enzyme mRNAs are lower than those of functionalization in other *Neotoma* species (Lamb et al., 2001). In addition, the eucalyptus specialist, the koala (*P. cinereus*), generates a higher number of oxidized metabolites than conjugation metabolites (Boyle et al., 2001; Pass et al., 2001). In the present study we found that in comparison to Sprague–Dawley rats, *N. stephensi* woodrats have a decreased capacity for glucuronidation, a major conjugation enzyme. For example, *N. stephensi* have decreased glucuronidation of *p*-nitrophenol, 1-napthol, and morphine substrates, in comparison to Sprague–Dawley rats.

One interesting result is the lack of detection of one of the major glucuronidation isoforms, UGT2B1, in *N. stephensi*. This result partially explains the decrease in UGT activity for morphine as this form of UGT is involved in morphine glucuronidation (King et al., 1997). The lack of UGT2B1 expression is in agreement with our hypothesis that specialist herbivores have a reduced expression of glucuronidation enzyme activity. Residual morphine UGT

activity is possibly due to additional UGT isoforms that have been shown to glucuronidate morphine (King et al., 1997). This effect seems to be unrelated to α-pinene since treatment of laboratory rats significantly increased UGT activity for morphine. The failure to detect UGT2B1 could also be due to a lack of sequence overlap between Sprague–Dawley rat probes used in the assay and the sequence for UGT2B1 in *N. stephensi*. However, we argue that the successful use of Sprague–Dawley rat probes to detect UGT2B1 in *N. lepida*, a sister species to *N. stephensi*, further suggests that *N. stephensi* may be lacking this enzyme. We are currently investigating the prevalence of UGT2B1 in additional species of woodrats.

It is possible that differences in enzyme activities and mRNA for detoxification enzymes between Sprague–Dawley rats and *N. stephensi* are attributable to gender differences. All the Sprague–Dawley rats used in the study were male, whereas all the *N. stephensi* were females. Although, differences in detoxification capability between genders exist, we do not believe that the sex of the woodrats confound our results (Guo et al., 1993). Furthermore, no previous studies have documented differences in the mRNA expression as large as those reported here due to sex. Thus, it is unlikely that gender is responsible for the detoxification differences.

Overall, data suggest that specialist mammalian herbivores have high functionalization enzyme activity and mRNA, and low expression of glucuronidation enzymes and mRNA compared to Sprague–Dawley rats. Increased expression of functionalization enzymes may result in more efficient detoxification of the specific secondary compounds specialists ingest in their limited diet. Similarly, several functionalization enzymes were enhance by exposure to α-pinene in Sprague–Dawley rats. However, conjugation enzymes were also enhanced in Sprague–Dawley rats treated with α-pinene. This result was not unexpected given that Sprague–Dawley rats are dietary generalists and that conjugation is used to metabolize a broad spectrum of toxins. We are currently testing the reliance of functionalization and conjugation pathways in additional specialists and generalist species of *Neotoma*.

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