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Xenobiotic metabolism of plant secondary compounds in juniper (*Juniperus monosperma*) by specialist and generalist woodrat herbivores, genus *Neotoma*

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Abstract

Mammalian herbivores routinely consume diets laden with often-toxic xenobiotics, yet the manner in which mammalian herbivores detoxify these plant secondary compounds (PSC) is largely unknown. Theory predicts that specialists rely more heavily on functionalization pathways whereas generalists rely on conjugation pathways to metabolize PSC in their diet. We took a pharmacological approach to determine how a specialist (*Neotoma stephensi*) of juniper foliage (*Juniperus monosperma*) and a generalist (*N. albigula*) may process the same dietary PSC. We investigated the xenobiotic metabolizing enzymes of the specialist and generalist on a control diet and a low (25%) juniper diet. We also examined enzyme activities in the specialist on a high (70%) juniper diet. We assayed for cytochrome P450 concentration and biotransformation activities of three specific cytochrome P450 isozymes (CYP1A, CYP2B, CYP3A), NAD(P)H:quinone oxidoreductase, glutathione conjugation, sulfation and glucuronidation. Results provide partial evidence for the hypothesis in that the specialist and generalist consuming juniper at a level similar to their natural diet, differ in the level of conjugation enzyme activity with generalists having higher activity overall than specialists.

Keywords: Conjugation; Cytochrome P450; Functionalization; Glucuronidation; Glutathione; Herbivore; *Neotoma*; Specialists

1. Introduction

The disparity in feeding strategies among herbivorous mammals is a paramount issue in the study of plant–mammal interactions. Evidence suggests that the inevitable challenge of consuming plant secondary compounds (PSC) governs the feeding strategies of many herbivores (Freeland and Janzen, 1974; Foley et al., 1999; Dearing et al., 2000). Many of the secondary compounds in plants are toxic and can cause cellular damage, prevent nutrient uptake or hinder growth (Bryant et al., 1992; Foley et al., 1995; Harju, 1996; Dearing and Cork, 1999). Most herbivorous mammals are generalized feeders and are capable of consuming low levels of a broad spectrum of PSC. Specialist herbivores, which consume large quantities of a limited spectrum of PSC, are extremely rare (Freeland and Janzen, 1974). The specialists that do exist consume levels of PSC that are toxic and sometimes lethal to most other mammalian herbivores. For example, the golden bamboo lemur (*Hapelemur aureus*) consumes a diet of a single species of bamboo equal to 12 times the lethal dose of cyanide per day for other lemurs (Glander et al., 1989).

More than three decades ago, Freeland and Janzen (1974) first suggested that the rarity of dietary specialization resulted from constraints in the mammalian detoxification system, limiting its capability to metabolize the large concentrations of PSC present in a diet of a single species of plant. Research thus far suggests that there are fundamental differences in the

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elimination of PSC by specialists and generalists. For example, specialists and generalists ingesting the same PSC have different physiological responses, excrete different metabolites in the urine and/or have different blood levels of PSC (Meyer and Karasov, 1989; Foley et al., 1995; Boyle et al., 2000, 2001; Mangione et al., 2000; Sorensen et al., 2001; Sorensen and Dearing, 2003). However, there are no comparative studies that have directly investigated the xenobiotic metabolizing enzymes and elimination strategies in closely related wild herbivores ingesting dietary PSC (Foley et al., 1999). The majority of research to date has been done comparing distantly related marsupials. Although much is known about the xenobiotic metabolizing processes of laboratory mammals because of pharmacological studies, this knowledge has not been extensively applied to studies of wild eutherian herbivores.

Mammalian xenobiotic metabolism is a two-phase system that transforms compounds into a hydrophilic form for elimination from the body in urine or bile (Parkinson, 1996). The process involves numerous "biotransformation enzymes" broadly divided into two classes: Functionalization (Phase I) and Conjugation (Phase II). Functionalization and conjugation have different physiological costs and benefits. Conjugation is thought to be more energetically expensive than functionalization but is physiologically safer. Functionalization reactions can produce reactive metabolites that bind protein or DNA, whereas conjugated metabolites tend to be less reactive (Morse and Stoner, 1993; Ayala and Cutler, 1996, 1997; Guengerich, 2006). Conjugation is predicted to incur a higher energetic cost than functionalization because it requires high-energy cofactors and the loss of the conjugated functional groups, such as glucose, sulfate and amino acids in the urine or bile. Several studies have demonstrated significant losses of energy in urine when animals consume a diet containing high levels of PSC (Cook et al., 1952; Foley and Hume, 1987; Sorensen et al., 2005).

It has been hypothesized that specialist herbivores rely more on functionalization enzymes than generalist herbivores to remain in energy balance (McLean et al., 1993, 2001; McLean and Foley, 1997; Boyle et al., 2001). Specialists must efficiently process high quantities of select PSC from diets low in nitrogen and digestible energy (Sorensen et al., 2005). It is predicted that specialists have restricted conjugation capabilities to minimize the loss of conjugated amino acids and carbohydrate products that are in limited supply in their diet. However, decreasing conjugation is not typically a physiologically safe strategy because conjugation protects against reactive metabolites produced by functionalization or accumulation of compounds not requiring functionalization (Morse and Stoner, 1993). The narrow diet of a specialist may permit reliance on functionalization if the metabolites formed during functionalization metabolism are not physiologically reactive. This increased dependence on functionalization of PSC would in theory reduce the cost of xenobiotic metabolism. In contrast, generalists are predicted to rely more heavily on conjugation than specialists. Although conjugation is expensive in terms of the cost of conjugates, the high diversity of plants ingested in the diet of a generalist compared to that of a specialist is thought to provide additional nutrients and energy required for conjugation. Conjugation pathways have broad-spectrum activities that safely biotransform the qualitatively diverse PSC in a generalist diet (Foley and McArthur, 1994; Parkinson, 1996).

To investigate the xenobiotic metabolizing mechanisms of generalist and specialist herbivores, we studied a pair of closely related herbivorous woodrats. These species are sympatric and the natural diets of each species, as well as tolerances to whole plants and specific PSC are well established (Vaughan, 1982; Dial, 1988; Dearing et al., 2000; Sorensen et al., 2005). The specialist woodrat consumes a diet primarily of *Juniperus monosperma* (75%) whereas the generalist consumes considerably less juniper (24%) as well as a variety of other plant species: *Yucca*, *Chrysothamnus*, *Rhus*, *Fallugia*, *Artemisia*, *Atriplex*, *Ephedra* (Dial, 1988). Laboratory trials corroborate that the specialist has the ability to consume twice as much juniper as the generalist. Furthermore, the specialist demonstrated a better performance in a number of physiological parameters than the generalist when both were consuming the diet of the specialist (Sorensen et al., 2001, 2005). The generalist's limited intake of juniper was not due to the poor nutritional qualities of juniper. Instead, the disparate feeding strategies appear to be the result of PSC in juniper (Dearing et al., 2000). Juniper contains high quantities of numerous terpenes and phenolics and therefore specialists presumably have developed efficient xenobiotic metabolizing mechanisms to mitigate the toxic effects of juniper.

The research presented here used *in vitro* pharmacological techniques to investigate the presence and degree of differences in specific xenobiotic metabolizing enzymes in the specialist, *Neotoma stephensi,* and the generalist, *N. albigula,* consuming control and juniper diets. We quantified enzyme activities of five principle xenobiotic metabolizing enzymes and three specific isozymes. Functionalization enzymes assayed included oxidation (cytochrome P450s) and reduction (NAD(P)H:quinone oxidoreductase). The cytochrome P450 (CYP) pathway is the most abundant and utilized functionalization pathway and consists of several isozymes with individual substrate specificities (Danielson, 2002). In addition to overall CYP content, three isozymes (CYP1A, CYP2B and CYP3A) were included either for their role in biotransformation of PSC in a variety of animal systems and/or because they are commonly quantified in pharmacological studies (Gregus et al., 1983; Rosenthal and Berenbaum, 1992; Hiroi et al., 1995; Parkinson, 1996; Pass et al., 1999, 2001; Danielson, 2002; Liukkonen-Anttila et al., 2003; Sivapathasundaram et al., 2003a). Conjugation enzymes analyzed were also selected for their common role in the biotransformation of a variety of compounds (Gregus et al., 1983; Rosenthal and Berenbaum, 1992; Sivapathasundaram et al., 2003b). Three conjugation enzymes were assayed: UDP-glucuronosyltransferase (UGT), glutathione transferase (GST) and sulfotransferase (SULT). UGT and GST represent the major conjugation enzymes in mammals (Dutton, 1980; Daniel, 1993). SULT is considered an important alternative to glucuronidation for phenolics and was included for this reason (Parkinson, 1996). The diversity of enzymes analyzed in this study is the most extensive to date for a pair of closely related mammalian herbivores.

2. Materials and methods

2.1. Study system

The study system consisted of a sympatric specialist–generalist pair from the southern Great Basin Desert in Arizona. Specialist (*Neotoma stephensi*) and generalist (*N. albigula*) woodrats were trapped near Wupatki National Park, 45 km NE of Flagstaff, AZ (35° 30′N, 111° 27′ W). Animals were transported to the University of Utah Animal Facility, housed in individual cages (48×27×20 cm) and put on a 12–12 hour light–dark cycle for at least 3 months prior to experiments to allow for Sin Nombre hantavirus screening. All animals were maintained on standard rabbit chow (Harland Teklad formula 2031) and water *ad libitum* prior to experimentation. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

2.2. Dietary treatments

Animals were fed one of three experimental diets (control, 25% juniper and 70% juniper). Control diet consisted of the same rabbit chow used for the maintenance diet in powdered form. *Juniperus monosperma* for the juniper diets was collected at trapping sites in Arizona by stripping foliage from several trees (>10) that had evidence of prior foraging by woodrats. Juniper was stored at −20 °C until use. Foliage was homogenized for the diet treatments by grinding it in a whirring blender with dry ice until it passed through a 1 mm screen. To prepare juniper diets, ground juniper was added to ground rabbit chow on a percent dry weight basis. The juniper treatments were prepared daily to minimize the volatilization of terpenes. Total food intake (g dry matter) was measured each day. For the control treatment, the specialist ($N=8$) and generalist ($N=12$) were fed the control diet of rabbit chow for five days to measure constitutive levels of xenobiotic metabolizing enzymes. The specialists ($N=4$) and generalists ($N=4$) in the low juniper treatment group were acclimated to a 5% juniper diet for two days and then placed on a 25% juniper diet for three days. The 25% juniper treatment represents the amount of juniper consumed by the generalist in nature and is also an amount on which the generalist and specialist can maintain weight. Only the specialist ($N=5$) was fed the high juniper treatment of 70% juniper diet. The generalists were not included in the 70% treatment because at that juniper concentration the generalist does not consume enough food to maintain body mass. For the high juniper treatment, specialists were first acclimated to 5% juniper for one day, followed by 25% juniper for one day, and treated with a 70% juniper for a final three days. Diets will be referred to as "low juniper" (25%) and "high juniper" (70%).

2.3. Microsomal preparation and enzyme assays

Enzyme activity assays were used to measure activity of hepatic enzymes among species and treatment groups. Enzyme activities were determined in sub-cellular fractions isolated from whole liver tissue. On the last day of each dietary treatment, animals were dispatched with CO_2. The liver was immediately perfused *in situ* by injecting cold isotonic saline into the hepatic portal vein and gall bladder was removed. The liver was then removed and weighed. Microsomal and cytosolic fractions were prepared by differential ultracentrifugation as described for laboratory rats by Franklin and Estabrook (1971) and stored at −80 °C until assayed for activity. Protein concentrations were determined colorimetrically by the method of Lowry et al. (1951) to standardize enzyme activity to protein concentration.

Overall CYP concentration was determined from microsomes using the carbon monoxide difference spectrum on a spectrophotometer by the method of Omura and Sato (1964) that measures the reduced P450:CO complex characteristic of all CYP isozymes cumulatively. CYP1A activities were determined by the method of Klotz et al. (1984) that measures the rate of resorufin production during the metabolism (*O*-deethylation) of 7-ethoxyresorufin (EROD; Sigma Chemical Co., St. Louis, MO, USA). CYP2B activities were determined by the method of Lubet et al. (1985) that measures the rate of resorufin production during the metabolism (de-alkylation) of pentoxyresorufin (PROD; Sigma Chemical Co., St. Louis, MO, USA). CYP3A activities were determined by testosterone 6$\tilde{\beta}$ hydroxylation (Sigma Chemical Co., St. Louis, MO, USA) where the testosterone metabolite (6$\tilde{\beta}$ hydroxylation) was separated by HPLC and quantified from its absorbance at 236 nm (Guengerich et al., 1986). Cytosolic NAD(P)H:quinone oxidoreductase (QOR) activities were determined by the dicoumarol-inhibited rate of reduction of 2, 6-dichlorophenolindophenol (Aldrich Chemical Co., Milwaukee, WI, USA) by NADH at pH 7.4 detected with spectrophotometry (Benson et al., 1980).

Hepatic microsomal UGT activities were determined with spectrophotometry (Bock et al., 1983) in reactions containing detergent (0.05% Triton X-100; Sigma Chemical Co., St. Louis, MO, USA) and measured the UDPGA-dependent disappearance rate of *p*-nitrophenol (Sigma Chemical Co., St. Louis, MO, USA). Cytosolic GST activities were determined with spectrophotometry (Habig and Jakoby, 1981), detecting the change in absorbance at 340 nm upon conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB:a general reference substrate; Aldrich Chemical Co., Milwaukee, WI, USA). SULT cytosolic activities were determined by detecting the PAPS-dependent disappearance rate of *p*-nitrophenol (Sigma Chemical Co., St. Louis, MO, USA) with spectrophotometry (Sekura et al., 1981).

The probe substrates used in the assays are considered isozyme specific. For example, isozyme CYP 1A catalyzes the metabolism of 7-ethoxyresorufin; SULT enzymes catalyze the metabolism of *p*-nitrophenol. Although these protocols were designed and validated in laboratory rats, they are often used in disparate species. Given that laboratory rats are in the same family as woodrats (*Neotoma*), the use of these assays likely provide a valid estimate of enzyme specific activities. It is worth noting that CDNB and *p*-nitrophenol are relatively nonspecific substrates for GST and UGT isozymes respectively. However, CDNB generally targets the alpha class of GST in liver assays and *p*-nitrophenol targets UGT1A's. The SULT substrate targets SULT1, used for phenol metabolism.

Table 1
Mean body mass (grams), dry matter intake (DMI) per gram of body mass (BM) and liver mass from woodrats on control diet, low juniper and high juniper diets†

N. albigula			*N. stephensi*		
Variable	Control diet	25% juniper	Control diet	25% juniper	70%juniper
Body mass	193.4±9.6	191.7±15.9	169±11.3	173.9±15.9	172.4±14.2
DMI per BM	0.12±0.005	0.12±0.008	0.12±0.006	0.11±0.008	0.10±0.007
Liver mass	6.17±0.6	7.60±1.0	5.56±0.7	6.75±1.0	4.45±0.9

†Data represented as means±1 SE.

2.4. Statistical analysis

The activities of each xenobiotic metabolizing enzyme were compared using a one-way ANOVA among all five treatment groups with diet being the main effect. A one-way ANOVA was used as opposed to a two-way because the 70% juniper specialist had no comparable group. When ANOVA analysis resulted in significance ($P<0.05$), we conducted a Post-Hoc Fisher's LSD for pair-wise comparison. Systat 10 (SPSS Inc., Chicago, IL, USA) was used for analysis.

3. Results

3.1. Body mass, food intake and liver mass

The final body mass of all treatment groups were not different (Table 1: ANOVA, $F_{4,\ 27}=0.944$ $P=0.454$). Relative food intake did not differ between the specialist and generalist treatment groups (Table 1: ANOVA, $F_{4,\ 27}=1.009$ $P=0.42$). Liver mass was not different among the specialist and generalist treatment groups (Table 1: ANOVA, $F_{4,\ 27}=1.656$ $P=0.189$).

3.2. Functionalization enzyme activity

3.2.1. Cytochrome P450 content

Overall, there were significant CYP concentration differences among the treatment groups (ANOVA, $F_{4,\ 28}=6.58$ $P=0.001$). CYP concentration levels did not differ constitutively between the specialist and generalist fed a control diet (Fig. 1). The differences arose from induction of CYPs on the juniper diets. CYP concentrations were significantly higher in the juniper diet treatments compared to the control diet treatments. The specialist and generalist treated with the low juniper diet both increased CYP concentration by approximately 50% over that of control diets. The specialist consuming a high juniper diet did not significantly increase CYP concentration from the low juniper diet (Fig. 1).

3.2.2. Quinone oxidoreductase

The levels of QOR activity (Fig. 1) were not significantly different in the specialist or generalist nor were they altered by any diet treatments (ANOVA, $F_{4,\ 28}=0.35$ $P=0.84$).

3.2.3. Cytochrome P450 isozymes

Activity levels for the specific isozymes CYP1A, CYP2B and CYP3A varied among the treatment groups (Fig. 2). CYP1A activities in the specialist fed each of the three dietary treatments (control, low and high juniper) were all less than half that of the generalist (ANOVA, $F_{4,\ 28}=6.58$ $P=0.005$). Neither the specialist nor generalist significantly increased CYP1A activity between control and juniper diets. CYP2B activities (Fig. 2) were not significantly different between the specialist and generalist fed the control or low juniper diet, but were increased nearly two-fold in the specialist fed the high juniper diet compared to all other groups (ANOVA, $F_{4,\ 28}=4.69$ $P<0.001$). Lastly, CYP3A activity levels were not different between the specialist and generalist fed control diet (Fig. 2). CYP3A activity was 37% higher when the generalist was fed a low juniper diet compared to control diet; however, this increase was not significant ($P=0.14$). CYP3A activities of the specialist did not differ across treatments (ANOVA, $F_{4,\ 28}=0.712$ $P=0.591$).

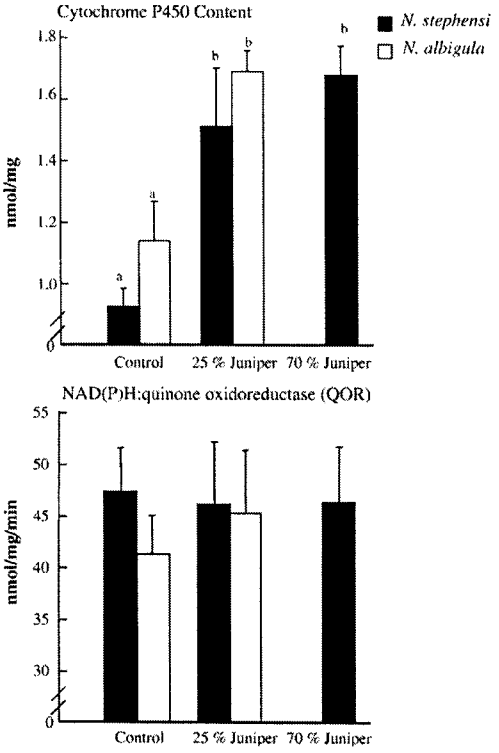

Fig. 1. Hepatic functionalization enzymes (mean+SE) in the specialist, *N. stephensi* (solid) and the generalist, *N. albigula* (open) fed diets containing different amounts of juniper. Data are represented as bars and different letters (a, b) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD.

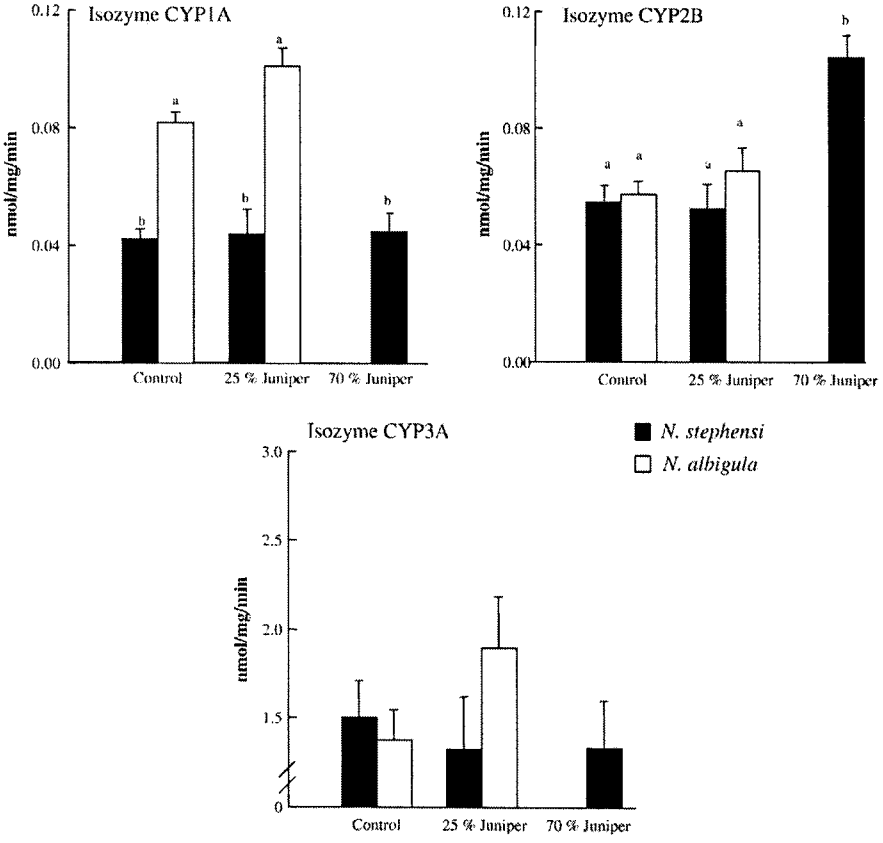

Fig. 2. Hepatic cytochrome P450 isozymes (mean+SE) in the specialist, *N. stephensi* (solid), and the generalist, *N. albigula* (open), fed diets containing different amounts of juniper. Data are represented as the mean+SE. Data are represented as bars and different letters (a, b) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD.

3.3. Conjugation enzyme activity

3.3.1. UDP-glucuronosyltransferases

There were overall significant differences in UGT activities among the treatment groups (ANOVA, $F_{4,\ 28}=10.94$ $P<0.001$). Constitutive UGT activities were not different between the specialist and generalist fed control diet (Fig. 3). When both the specialist and generalist were fed a low juniper diet, each significantly increased activity from control, with the specialist reaching higher levels than the generalist. In contrast, UGT activity of the specialist on the high juniper treatment was lower than that of the low juniper treatment and equivalent to constitutive levels.

3.3.2. Sulfotransferase

Levels of SULT activity were significantly different between the specialist and generalist (ANOVA, $F_{4,\ 28}=5.67$ $P=0.002$). SULT activity in the generalist was approximately two and a half times higher than the specialist in all dietary treatments. There was no change in SULT activity by either the specialist or the generalist from control diet to juniper diets (Fig. 3).

3.3.3. Glutathione transferase

Overall levels of GST activity were different among treatment groups (ANOVA, $F_{4,\ 28}=3.50$ $P=0.019$). Levels of constitutive GST activity were not different between the specialist or the generalist fed control diet (Fig. 3). However, there was a significant increase between the generalist on the control diet and low juniper diet. In the specialist, the high juniper diet significantly decreased GST activity from that on either the control or the low juniper diets.

4. Discussion

It has been postulated that because of dietary differences and energy balance requirements, specialist herbivores might rely to a greater extent on less energetically draining functionalization enzymes to eliminate PSC from their diet while generalists may utilize conjugation enzymes that require high-energy cofactors to a greater extent (McLean et al., 1993, 2001; McLean and Foley, 1997; Boyle et al. 2001). Results of the functionalization enzymes assayed were not consistent with the hypothesis that specialists rely more heavily on functionalization. The specialist

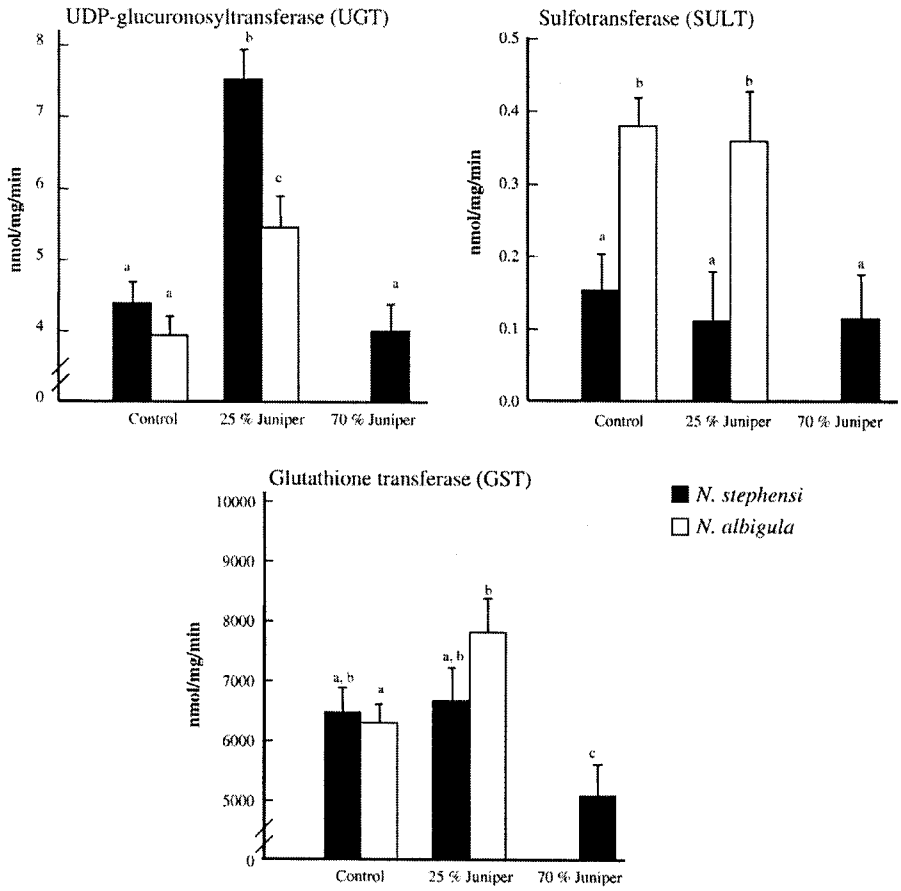

Fig. 3. Hepatic conjugation enzymes (mean + SE) in the specialist, *N. stephensi* (solid), and the generalist, *N. albigula* (open), fed diets containing different amounts of juniper. Data are represented as the mean + SE. Data are represented as bars and different letters (a, b, c) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD.

and generalist appeared to have the same activity levels of functionalization enzymes (CYP content and QOR) on a juniper diet (Fig. 1). However, the specific CYP isozymes assayed differed in activity (Fig. 2). Results from the conjugation enzymes assayed were partially consistent with this hypothesis in that the generalist had higher activities of two of the three conjugation enzymes assayed and had constitutively higher levels of the third enzyme compared to the specialist when both are fed an ecologically relevant diet. However, this is only seen when comparing the specialist on the ecologically relevant high juniper (70%) diet. The specialist fed low juniper (25%) diet had elevated UGT activity compared to the generalist on the same diet. When the specialist consumed a high concentration of juniper comparable to its natural diet, its levels of UGT activity dropped to constitutive levels. Furthermore, the high juniper fed specialist's GST activity was significantly lower than constitutive levels. In the subsequent paragraphs, we compare constitutive and induced differences in functionalization enzymes followed by conjugation enzymes. The concluding paragraphs evaluate the implications and potential explanations for differences in xenobiotic metabolizing enzyme activities.

4.1. Functionalization

Approximately a decade ago, it was postulated that *Eucalyptus* specialists, such as the koala (*Phascolarctos cinereus*), rely more on functionalization reactions than generalists (McLean and Foley, 1997). Studies on *Eucalyptus* specialists and generalists supported this paradigm. For example, specialists (koala, greater glider (*Petauroides volans*), ringtail possum (*Pseudocheirus peregrinus*)) exposed to the two main PSC in *Eucalyptus* appeared to use functionalization to a greater extent than generalists (brushtail possum (*Trichosurus vulpecula*) and laboratory rat) as indicated by more extensively oxidized urinary metabolites excreted by specialists (McLean and Foley, 1997; Boyle et al., 1999, 2001). The suite of xenobiotic metabolizing enzymes used by these specialists and generalists is unknown as the research was based on structures of metabolites in the urine rather than individual enzyme activities. Furthermore, results from two studies measuring CYP content in a range of marsupials and the laboratory rat found lower concentrations in generalists compared to the koala, with the exception of the Quokka (*Setonix brachyurus*) and brushtail possum (Bolton and

Ahokas, 1997; Stupans et al., 1999). To date, pharmacological data has accumulated, mostly with regard to koala metabolism, that suggest the koala relies on functionalization enzymes, particularly cytochrome P450s, as well as cytosolic alcohol and aldehyde dehydrogenases (Stupans et al., 2001). Research targeting xenobiotic metabolism of specialist mammals has mostly been restricted to marsupial *Eucalyptus* specialists with very little described in other specialist systems.

Based on the marsupial work, we predicted that the specialist, *N. stephensi*, would also have higher constitutive or induced response to juniper in functionalization enzyme activities compared to the generalist, *N. albigula*. However, the specialist and generalist did not differ in QOR activity irrespective of diet (Fig. 1). Furthermore, CYP content was induced equally among both the specialist and generalist on all juniper diet treatments (Fig. 1). CYP content of the specialist woodrat consuming a juniper diet was similar to that measured in woodrats only days after capture from nature where they were presumably consuming a diet containing approximately 70% juniper (Stupans et al., 1999; Lamb et al., 2004).

Though induction of CYP concentration was equal, activities of specific CYP isozymes differed between the specialist and generalist (Fig. 2). In this study, activities of CYP1A in the specialist were constitutively one-half that of the generalist compared among all diet treatments. A second CYP isozyme, CYP2B, varied among diet treatments in the specialist but not the generalist. The specialist had a nearly two-fold induction of CYP2B activity when fed the high juniper diet. The levels of CYP2B activity measured in the specialist on the high juniper diet are consistent with that measured in woodrats only days after capture from juniper woodlands (Lamb et al., 2004). The increased levels of CYP2B activity in the specialist may play a key role in providing the capability of the specialist to metabolize the quantity of terpenes found in high levels of juniper. CYP3A activity was not different between the specialist and generalist on the dietary treatments.

The induction of CYPs by terpenes in rat liver microsomes has previously been reported. A number of studies have shown terpenes to induce CYP2B. Two studies investigating the effect of various terpenes, including α-pinene (the principle terpene in juniper), documented induced CYP2B several-fold as measured by immunoassays (Austin et al., 1988; Hiroi et al., 1995). Furthermore, CYP2B activity increased when laboratory rats were given an oral dose of α-pinene (Lamb et al., 2004). Evidence suggests that CYP3A metabolizes some terpenes. CYP3A oxidizes 1,8-cineole in rats and humans (Miyazawa et al. 2001). CYP3A in laboratory rats was induced upon exposure to terpenes, 1,8 cineole and cadinene, but there was no induction with α-pinene, consistent with the absence of increased CYP3A activity in woodrats consuming a juniper diet reported here (Hiroi et al., 1995).

4.2. Conjugation

Previous research on mammalian herbivores indicates that generalists have higher levels and a more diverse array of xenobiotic metabolizing enzymes to process the myriad PSC in a varied diet than specialists. Prior studies on *Eucalyptus* specialists and generalists found a more diverse array of urinary metabolites in generalists compared to specialists (Boyle et al., 1999) suggesting the involvement of a greater number of enzymes.

Results from this research are partially consistent with the prediction that generalists rely more on conjugation enzymes for elimination of PSC compared with specialists. While overall the generalist demonstrated induction of two of three conjugation enzymes and constitutively higher levels of the third enzyme, the specialist did exhibit a greater level of induction of UGT compared to the generalist. In the generalist, SULT activity was constitutively higher. Furthermore, the generalist increased both GST and UGT activities on the juniper diet. In contrast, the specialist only increased UGT in response to low juniper diet, however to a greater extent than the generalist.

The overall pattern of conjugation enzyme activity when both the specialist and generalist were fed ecologically relevant diets (generalist fed 25% juniper; specialist fed 70% juniper) provides evidence that the specialist may reduce the activity of conjugation enzymes for elimination of PSC compared with the generalist. The specialist reduced the activity of the conjugation enzymes assayed compared to generalists on a low juniper diet when the concentration of juniper was increased to an ecologically relevant diet of 70% juniper. The specialist fed the high juniper diet reduced activity of UGT to levels similar to those on the control diet (Fig. 3). Furthermore, GST levels appeared to be suppressed in the specialist on the high juniper diet in that they were reduced to levels significantly lower than those on the control and low juniper diets. SULT activities of the specialist were not affected by a juniper diet and remained consistently at half that of the generalist. In summary, the specialist on a high juniper diet exhibited constitutive levels of UGT and SULT and suppressed levels of GST activity (Fig. 3). The reduction in UGT and GST enzyme activities of the specialist from that of the low juniper treatment was likely a change in cellular regulation of enzyme expression caused by the change in PSC dose.

A previous study (Lamb et al., 2004) measuring levels of similar xenobiotic metabolizing enzymes in recently caught *N. stephensi* found levels of UGT activity higher than those measured in the specialist on a high juniper diet. The assumption was that the recently caught specialist was consuming, in the wild, a diet containing high levels of juniper. Activities should, therefore, be similar to specialists consuming the high juniper diet in this study. The other enzyme assays measured in both of these studies, such as CYP2B, had similar activity levels (Lamb et al., 2004). Differences may be due to duration of exposure to juniper in that long-term exposure and short-term exposure can alter enzyme activities. However, three days exposure is recognized in pharmacological studies as sufficient time to fully induce enzyme activity. Otherwise, during transport the wild caught specialist may have taken in a reduced amount of juniper and therefore expressed activity levels of UGT more similar to those fed a low juniper diet (van der Logt et al., 2003). This difference in UGT activities between these studies requires further work for clarification.

4.3. Ecological implications

The extreme energetic expense of the xenobiotic metabolism of PSC, on par with reproduction, thermoregulation or growth, is an inevitable consequence of herbivory (Sorensen et al., 2005). Most herbivores are unable to increase food intake to compensate for the cost of xenobiotic metabolism because of the concomitant increase in PSC. Minimizing conjugation is possibly a mechanism employed by specialists to conserve the carbohydrates, amino acids and energy used to make "high energy" co-substrates that would otherwise be lost as conjugates in the urine or feces. Instead, CYP2B may be relied upon more strictly for metabolism of juniper terpenes by the specialist. Additionally, there may be other 'low-cost' enzymes involved that are worthy of more investigation. In laboratory feeding trials, the specialist on a juniper diet had twice the energy available for activities such as reproduction compared to the generalist (Sorensen et al., 2005). The dose-dependent change in UGT and GST conjugation, as well as the constitutively lower levels of SULT, may explain this phenomenon. UGT and GST conjugation are characterized by high capacity and broad specificity but may also result in significant energetic expense. In energetic studies of juniper consumption, the generalist expended significantly more of its energy budget on the excretion of glucuronic acid compared to the specialist (Sorensen et al., 2005). In our study, the specialist reduced UGT enzyme activity on 70% juniper diets compared to 25% juniper diet. Furthermore, GST conjugation activity levels were suppressed compared to control and low juniper diets. This may be a physiological mechanism to reduce the energetic cost of xenobiotic metabolism.

This study is the first report that specialists change xenobiotic metabolizing enzyme activities at different concentrations of dietary PSC. These concentration dependent differences may allow specialists to use conjugation enzymes during periods of the year when they opportunistically consume plants other than juniper. Previous field studies indicate that from May through September specialists decrease their intake of juniper to 65% and increase that of other plants (Dial, 1988). The plant species consumed during this period are more nutrient rich than juniper and thus could provide additional glucose substrates needed for glucuronidation.

The mechanism by which specialists and generalists differ in their ability to eliminate plant toxins is a fundamental topic in both mammalian and insect herbivore research. This research expands on previous comparative research with a new study system that is eutherian and provides one of the most thorough profiles of xenobiotic metabolizing enzymes in closely related wild mammalian herbivores. Increased CYP2B activity and a reduction in conjugation activities, in particular GST, may play a major role in facilitating the metabolism of the quantities of terpenes found in a high juniper diet. However, there are likely other enzymes involved that were not analyzed in this research. The reduced activity of conjugation enzymes assayed in this research emphasizes the need to explore other enzymes, particularly functionalization enzymes, which may be involved in metabolism of a high terpene diet. We are currently investigating a broad array of both functionalization and conjugation enzymes used by the specialist and generalist using microarray technology. This technology will permit an even more extensive analysis of the types of enzymes expressed by specialists and generalists.

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